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## Recent Developments in the Application of Baeyer–Villiger Monooxygenases as Biocatalysts

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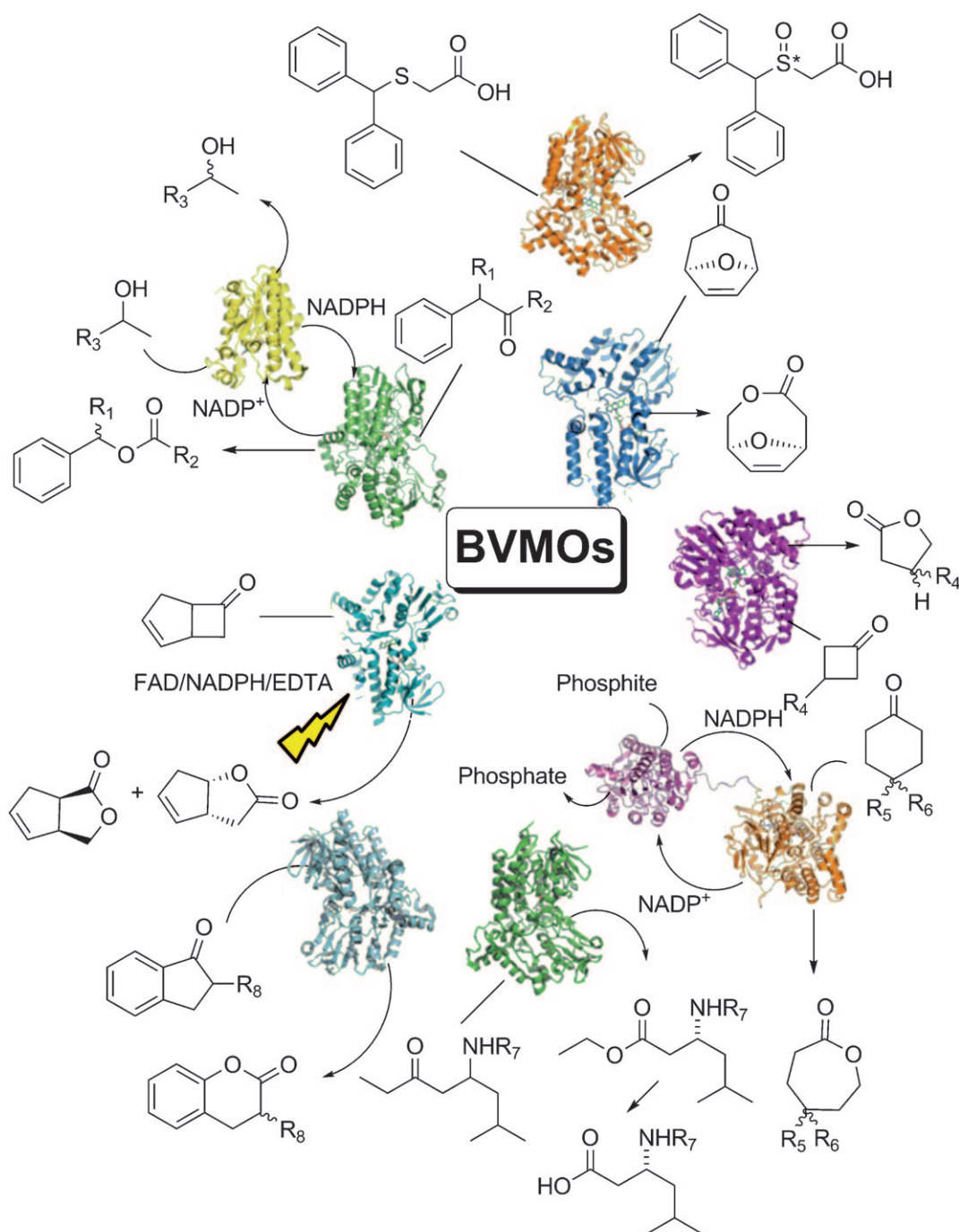
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Gonzalo de Gonzalo,<sup>\*,[a]</sup> Marko D. Mihovilovic,<sup>[b]</sup> and Marco W. Fraaije<sup>\*,[a]</sup>

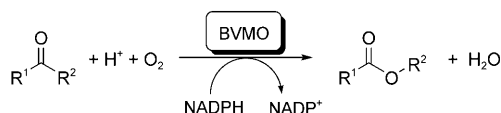
Baeyer–Villiger monooxygenases (BVMOs) represent a specific class of monooxygenases that are capable of catalyzing a variety of oxidation reactions, including Baeyer–Villiger oxidations. The recently elucidated BVMO crystal structures have provided a more detailed insight into the complex mechanism of these flavin-containing enzymes. Biocatalytic studies on a number of newly discovered BVMOs have shown that they are very potent oxidative biocatalysts. In addition to catalyzing the regio- and enantioselective Baeyer–Villiger oxidations of a wide

range of carbonylic compounds, epoxidations, and enantioselective sulfoxidations have also been shown to be part of their catalytic repertoire. This review provides an overview on the recent developments in BVMO-mediated biocatalytic processes, identification of the catalytic role of these enzymes in metabolic routes and prodrug activation, as well as the efforts in developing effective biocatalytic methodologies to apply BVMOs for the synthesis of high added value compounds.

## 1. Introduction

### Baeyer–Villiger monooxygenases fulfil various roles in nature

A large variety of enzymes have evolved for selective oxidations in nature that include as major enzyme classes oxidases,<sup>[1]</sup> dehydrogenases,<sup>[2]</sup> monooxygenases,<sup>[3]</sup> dioxygenases,<sup>[4]</sup> and peroxidases.<sup>[5]</sup> Within the class of monooxygenases a subclass of enzymes is able to catalyze Baeyer–Villiger oxidations (Scheme 1).<sup>[6]</sup>



**Scheme 1.** Baeyer–Villiger oxidation of carbonylic compounds catalyzed by BVMOs.

Baeyer–Villiger monooxygenases (BVMOs, EC 1.14.13.x) were first isolated in the 1960s,<sup>[7]</sup> and the first BVMO-encoding genes were identified in the 1990s.<sup>[8]</sup> The majority of all studied BVMOs are sequence related (type I BVMOs), belong to the subclass B flavoprotein monooxygenases,<sup>[9]</sup> carry a FAD cofactor as prosthetic group, and depend on NADPH as electron donor. All type I BVMOs described so far are of microbial origin. Such prevalence in microbes was confirmed by analyzing genome sequences. By using a BVMO-specific protein sequence motif, BVMOs can effectively be annotated and on average about one BVMO gene is found in each microbial genome.<sup>[10]</sup> However, the genomic distribution is quite special: BVMOs are abundant in some specific microbes, for example, actinomycetes and some filamentous fungi, whereas they are hardly represented in other types of bacteria and appear to be fully absent in higher eukaryotes and archaea. Along with the observation that microbes harbor a multitude of unexplored BVMOs, research in the past few decades has revealed that these BVMOs are employed for a wide variety of functions by microbes. They are involved in catabolic pathways enabling bacteria to grow on a variety of ketones,<sup>[11]</sup> and they are also crucial in the biosynthesis of a variety of secondary metabolites.<sup>[12]</sup> Although a dedicated BVMO has evolved for each of these metabolic steps, biocatalytic studies have also shown that individual BVMOs can accept a formidable number of different substrates. For the most extensively studied BVMO, cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 (CHMO<sub>Acineto</sub>; EC 1.14.13.22), it has been shown

that it can convert hundreds of different compounds.<sup>[13]</sup> Except for performing Baeyer–Villiger oxidations, this enzyme has also been used to demonstrate the capability of BVMOs to catalyze other oxidation reactions: for example, sulfoxidations, oxidations of selenium and boron-containing compounds, epoxidations, and N-oxidations. Such catalytic versatility has also been observed for other BVMOs and is consistent with the proposed mechanism.<sup>[14]</sup> BVMOs use a flavin cofactor for catalysis that, after coenzyme-mediated reduction and reaction with molecular oxygen, forms a peroxyflavin intermediate. Such an enzymatic peracid analogue can perfectly explain the broad range of oxidation reactions that are feasible with BVMOs. Kinetic studies have revealed that the formation of this intermediate does not depend on substrate binding.<sup>[15]</sup> Therefore, the reactive peroxyflavin intermediate, as a cocked gun, is just waiting for a substrate to reach the active site. The accessibility of the active site will determine the substrate range of each BVMO. The recently reported BVMO crystal structures have shown that during individual catalytic steps major structural rearrangements occur.<sup>[16]</sup> Because no crystal structure has yet been obtained of a BVMO in the peroxyflavin state, the exact molecular basis for the substrate specificity and regio- and/or enantioselectivity of BVMOs is still unclear. Nevertheless, the recent studies have provided a wealth of information on unique catalytic properties of natural or engineered BVMO, often offering catalytic strategies that are impossible by using chemical means. This has led to an increased interest in employing BVMOs as synthetic tools or as (pro)drug targets. This review provides an overview on the recent developments in BVMO-based catalytic applications. It will focus on recently reported biocatalytic approaches that involve BVMOs, examples of BVMOs involved in synthesis of bioactive molecules, and recent progress in developing effective strategies to apply BVMOs.

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## 2. BVMO-Mediated Chemo- and/or Regioselective Oxidations and Desymmetrization of Prochiral Substrates

### CHMO and CPMO: similar and different

The biocatalytic potential of CHMO<sub>Acineto</sub><sup>[14,17]</sup> and cyclopentanone monooxygenase from *Comamonas* sp. NCIMB 9872 (CPMO<sub>Coma</sub>; EC 1.14.13.16)<sup>[8a,18]</sup> have been intensely studied. Because of their broad substrate tolerance and high chemo-, regio-, and/or enantioselectivity, they are highly attractive bio-

Gonzalo de Gonzalo obtained his Ph.D. (2003) under Prof. Vicente Gotor at the University of Oviedo. He worked with Dr. Giacomo Carrea at the Istituto di Chimica del Riconoscimento Molecolare (Milan, Italy) before returning to the University of Oviedo. Currently, he is spending one year at the University of Groningen in the research group of Prof. Marco W. Fraaije. His research focuses on the study and application of biocatalytic methodologies to the synthesis of high-added-value compounds and to the development of new biocatalysts.



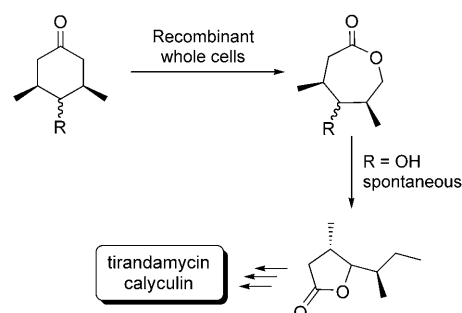
Marko Mihovilovic obtained his Ph.D. in 1996 (Prof. Peter Stanetty, Vienna University of Technology). He performed post-doctoral research as an Erwin Schrödinger Fellow in the laboratories of Prof. Margaret Kayser (University of New Brunswick, Canada) and Prof. Jon Stewart (University of Florida, USA). He returned to the Vienna University of Technology to complete his Habilitation in 2003. He is currently Associate Professor at the Institute of Applied Synthetic Chemistry where his group is active in several fields of bioorganic synthesis.



Marco W. Fraaije did his Ph.D. at Wageningen University (Prof. Willem van Berkel). After an EMBO postdoctoral fellowship in protein crystallography (University of Pavia, Prof. Andrea Mattevi), he moved to the University of Groningen to join the group of Prof. Dick Janssen. He became an Associate Professor in 2007, leading a research group in molecular enzymology. His research focuses on the discovery and redesign of flavoprotein oxidases and Baeyer–Villiger monooxygenases, but also aims to better understand the sequence–function relationship of redox enzymes.



catalysts. Comparative biocatalytic studies have recently revealed that both BVMOs, although highly similar in sequence (37 % seq. identity), can be regarded as prototypes of two distinct BVMO subclasses.<sup>[19]</sup> For a number of substrates tested, it was observed that catalysis by CHMO<sub>Acineto</sub> or CPMO<sub>Coma</sub> led to opposite regio- and/or stereoisomers as products.<sup>[20]</sup> This divergence of CPMO or CHMO-like enzymes was confirmed when using a set of BVMOs that display significant sequence identity with one of the two prototypes. Thus, two enzymatic clusters were established for these enzymes. The CHMO-type group is formed by CHMO<sub>Acineto</sub> and the CHMOs from *Arthrobacter* (CHMO<sub>Arthro</sub>),<sup>[21]</sup> *Brachymonas* (CHMO<sub>Brachy</sub>),<sup>[21b]</sup> *Rhodococcus* (CHMO<sub>Rhodo1</sub> and CHMO<sub>Rhodo2</sub>),<sup>[21b,c,22]</sup> and *Brevibacterium* HCU (CHMO<sub>Brevi1</sub>),<sup>[23]</sup> whereas the CPMO-type family consists of CPMO<sub>Coma</sub> and another BVMO from *Brevibacterium* (CHMO<sub>Brevi2</sub>).<sup>[24]</sup> Recently, *Escherichia coli* expressing the two prototype biocatalysts, CHMO<sub>Acineto</sub> and CPMO<sub>Coma</sub>, were used for the oxidation of a set of 4-substituted 3,5-dimethylcyclohexanones (Scheme 2 and Table 1).<sup>[25]</sup> All tested ketones were converted into the corresponding lactones with good yields and high optical purities when employing CHMO<sub>Acineto</sub>. Only the oxidation of 3,5-dimethyl-4-methylenecyclohexanone was performed with a slightly decreased enantioselectivity. In contrast, CPMO<sub>Coma</sub> was only able to oxidize this latter compound and 3,5-dimethylcyclohexanone. For both compounds, excellent enantioselectivities were obtained in the formation of the



**Scheme 2.** BVMO-catalyzed oxidation of 4-substituted-3,5-dimethylcyclohexanones employing CHMO<sub>Acineto</sub> and CPMO<sub>Coma</sub>.<sup>[25]</sup>

**Table 1.** Biotransformation of 4-substituted-3,5-dimethylcyclohexanones catalyzed by CHMO<sub>Acineto</sub> and CPMO<sub>Coma</sub>-expressing recombinant cells.<sup>[25]</sup>

R	Enzyme	Yield [%] <sup>[a]</sup>	ee [%] <sup>[b]</sup>	[α] <sub>D</sub> <sup>20</sup>
cis-OH	CHMO	77	99	−24.5 (1.71, CHCl <sub>3</sub> )
cis-OH	CPMO	n.c. <sup>[c]</sup>	n.a. <sup>[d]</sup>	n.a. <sup>[d]</sup>
trans-OH	CHMO	80	96	+53.7 (1.76, CHCl <sub>3</sub> )
trans-OH	CPMO	n.c. <sup>[c]</sup>	n.a. <sup>[d]</sup>	n.a. <sup>[d]</sup>
=CH <sub>2</sub>	CHMO	54	92	+8.4 (0.80, CHCl <sub>3</sub> )
=CH <sub>2</sub>	CPMO	63	99	−8.6 (1.00, CHCl <sub>3</sub> )
H	CHMO	65	99	−12.7 (2.00, CHCl <sub>3</sub> )
H	CPMO	58	91	+7.7 (1.30, CHCl <sub>3</sub> )

[a] Yield after purification by column chromatography. [b] Determined by GC. [c] No conversion. [d] Not applicable.



lactones, which again were found to be antipodal to the ones obtained with CHMO<sub>Acinetor</sub>. The oxidation of the *cis*-4-hydroxy-3,5-dimethylcyclohexanone led to the corresponding lactone, which suffers a rearrangement process leading to a chiral butyrolactone that is a key intermediate in the synthesis of calyculin and tirandamycin A.

A set of bridged cycloketones has also been synthesized and employed as substrates by using CHMO and CPMO-type BVMOs.<sup>[26]</sup> Interestingly, four to six stereogenic centers can be created by desymmetrization of these ketones. For almost all the ketones tested, again, enantiodivergent behavior of both BVMO subclasses was observed. Thus, CHMO-type BVMOs led to the (+)-lactones, whereas oxidations catalyzed by CPMO<sub>Coma</sub> and CHMO<sub>Brevi2</sub> afforded the (–)-lactones.

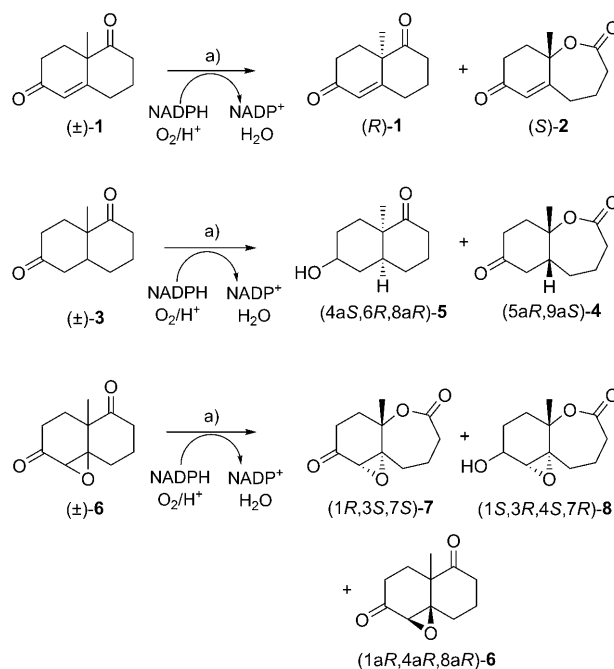
The biocatalyzed Baeyer–Villiger oxidation of 4,4-disubstituted cyclohexanones, that had been previously performed by using CHMO<sub>Acinetor</sub><sup>[27]</sup> has recently been tested with a larger set of CHMO- and CPMO-type BVMOs.<sup>[28]</sup> Most of the BVMOs employed were found to effectively convert 4,4-dimethylcyclohexanone, with the exception of CHMO<sub>Brevi1r</sub>, which showed low reactivity for all these substrates. Biotransformation of 4-ethyl-4-methylcyclohexanone led to the corresponding lactone with good yields and enantiomeric excesses around 80% for the CHMO-family, whereas the CPMO-type enzymes displayed much lower selectivity while yielding the same enantiomer of the lactone. Biooxidation of 4-hydroxy-4-methylcyclohexanone resulted in the enantiodivergent formation of both lactones depending on the employed CHMO or CPMO-type enzyme. With the CHMO-type enzymes the synthesis of the (–)-lactone was possible, whereas, with only moderate enantiomeric excesses, the (+)-lactones were obtained when using a CPMO-type BVMO. The final 7-membered ring product undergoes a spontaneous rearrangement to afford the more stable 5-membered ring lactone. Recently, the CHMO from *Xanthobacter* sp. XL5, cloned in 2003 and classified as a CHMO-type BVMO,<sup>[22]</sup> has been employed for the oxidation of 4,4-disubstituted cyclohexanones, leading to the corresponding (–)-lactones with good yields and high enantiomeric excesses.<sup>[29]</sup> This enzyme was able to catalyze the oxidation of 4-methyl-4-phenylcyclohexanone, which was not a substrate for the rest of the recombinant BVMOs tested.

Both BVMO-subclasses have also been employed for the enzymatic Baeyer–Villiger oxidation of a set of prochiral alkyl di-substituted perhydropyran-4-ones.<sup>[30]</sup> Previous experiments had established that the oxidation of perhydropyran-4-one occurred with high yields for all the enzymes tested.<sup>[31]</sup> The presence of different alkyl chains led to a remarkable difference in substrate acceptance: CHMO-type enzymes displayed a high efficiency and selectivity in the oxidation of alkyl chains up to three linear carbon atoms to obtain the final (2*S*,7*R*)-lactones, whereas the presence of more branched substituents or longer chains was not accepted by CHMOs. The CPMO-group was not able to convert all of these substrates. However, CPMOs were found to oxidize constrained bicycloperhydropyran-4-ones leading to the corresponding (1*S*,6*S*)-lactones with moderate yields and high enantiomeric excess. Such conversion was not observed for the CHMO-type enzymes.

## Exploring chemoselectivity of BVMOs

Although it has been shown that CHMO<sub>Acinetor</sub> can be used to perform Baeyer–Villiger oxidation with a variety of ketones, in particular by focussing on achieving good regio- and/or enantioselectivity, little attention has been paid to explore possible chemoselectivity. Only a few studies on substrates presenting more than one group susceptible to enzymatic oxidation have been conducted. It has been shown some years ago, that oxygenation proceeds preferably at the carbonyl group in the presence of other oxidizable functional groups (alkenes, thioethers).<sup>[30–32]</sup> Recently, the chemoenzymatic Baeyer–Villiger oxidation of bicyclic diketones catalyzed by a crude preparation of CHMO<sub>Acinetor</sub> expressed in *E. coli* has been employed to synthesize different ketolactones, compounds that are of interest as key intermediates in natural products.<sup>[33]</sup>

The CHMO-catalyzed oxidation of the Wieland–Miescher ketone (1) was completely selective to generate the corresponding enantiopure (*S*)-ketolactone, (*S*)-2 from the saturated carbonyl group, whereas the unreacted (*R*)-diketone was recovered in 43% yield and 80% enantiomeric excess (Scheme 3). Racemic 8a-methylhexahydronaphthalene-1,6-dione (±)-3 was chemoselectively oxidized to give the (5*aR*,9*aS*)-ketolactone 4 with excellent enantiomeric excess. Formation of 32% of enantiopure ketoalcohol 5 was observed due to the presence of a dehydrogenase in the BVMO preparation. Chemical epoxidation of the racemic Wieland–Miescher ketone led to an epoxidione 6, which is transformed by CHMO catalysis into the enantiopure lactone (1*R*,3*S*,7*S*)-7 as the only Baeyer–Villiger product. This conversion also afforded 32% of the unaltered starting (1*aR*,4*aR*,8*aR*)-epoxidione with 95% optical purity and an 11% of enantiopure ketoalcohol 8 generated again by a dehydrogenase activity on the starting material.

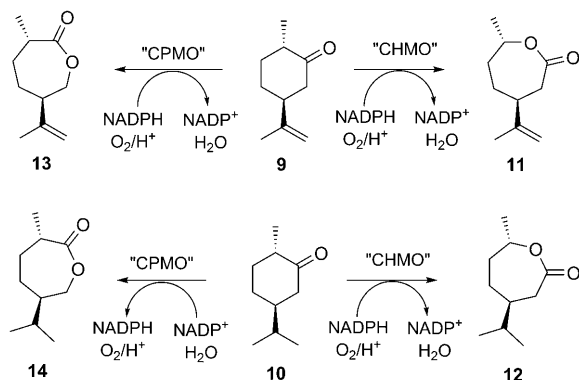


**Scheme 3.** CHMO<sub>Acinetor</sub>-catalyzed chemoselective oxidation of bicyclic ketones.<sup>[33]</sup> a) Tris-HCl, 50 mM, CHMO<sub>Acinetor</sub> G6P/G6PDH.

## BVMOs govern regioselectivity

The regioselectivity of the Baeyer–Villiger oxidation is governed by steric, conformational and electronic effects typically leading to migration of the more-substituted (the more nucleophilic) carbon center for chemical oxidations. Such migratory aptitude is also seen in the majority of the enzyme-catalyzed Baeyer–Villiger oxidations. In some cases, however, the use of BVMOs has led to the formation of the “unexpected” or “abnormal” lactones. This underscores the unique properties of BVMO that can be of use in synthetic chemistry. It is suggested that in these cases the chiral environment of BVMOs imposes restrictions that determines which moiety will migrate, as certain stereoelectronic requirements have to be met in order to allow for the rearrangement process. As a result, in several cases BVMOs have been shown to afford one of the regioisomerically possible lactones that might be different from the “expected” or “normal” lactone. In the last few years, several atypical regioselective Baeyer–Villiger oxidations have been described by using BVMOs as catalysts.

The collection of CHMO and CPMO-type BVMOs expressed in recombinant *E. coli* strains has been applied for the regiodivergent biooxidation of different terpenones.<sup>[34]</sup> Baeyer–Villiger reaction of (–)-*trans*-dihydrocarvone (**9**) and (–)-carvomenthone (**10**) catalyzed by CHMO-type enzymes led to the formation of the expected lactones (**11** and **12**, respectively) with good to excellent conversions. However, the opposite enantiomers (+)-*trans*-dihydrocarvone and (+)-carvomenthone were transformed into the (+)-unexpected lactones, whereas the use of CPMO-type biocatalysts led in all cases to the exclusive formation of the unexpected compounds (**13** and **14**), albeit with low conversions (Scheme 4). (–)-*cis*-Dihydrocarvone was



**Scheme 4.** Regiodivergent oxidation of (–)-*trans*-dihydrocarvone and (–)-carvomenthone by employing different BVMOs.<sup>[34]</sup>

converted to the expected lactone in high yields by CHMO-type BVMOs, whereas the CPMO enzymes afforded the unexpected products. The (+)-*cis* enantiomer was converted with low regioselectivities by all the BVMOs tested.

CHMO and CPMO-type enzymes have also been employed for the biooxidation of a set of fused cyclobutanones.<sup>[35]</sup> This again revealed divergent catalytic behavior: the CHMO-type enzymes are able to oxidize these compounds to form both

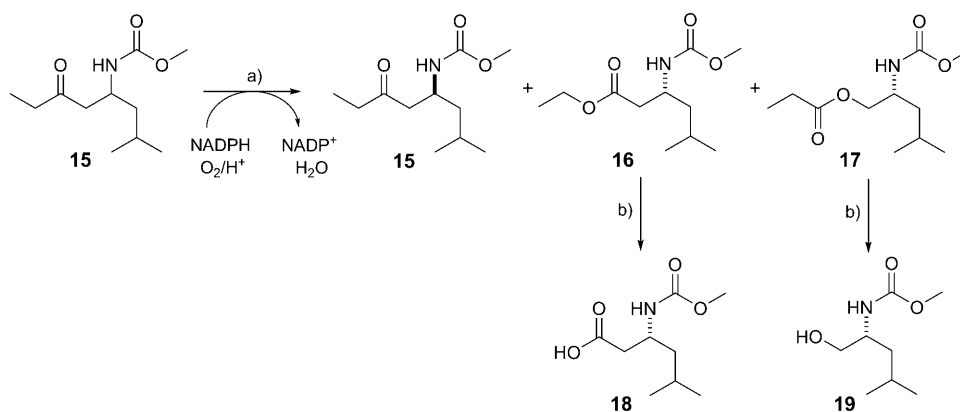
possible regioisomeric lactones with high optical purities, whereas the CPMO-type BVMOs led to the formation of the expected lactones as the only regioisomer. Thus, the two BVMOs subclasses can nicely complement each other for covering a broad range of reactions. Remarkably, a recently characterized BVMO from *Mycobacterium tuberculosis* displayed preferred formation of the atypical lactone in a kinetic-resolution process.<sup>[36]</sup>

*E. coli* cells expressing 4-hydroxyacetophenone monooxygenase (HAPMO; EC 1.14.13.81)<sup>[37]</sup> from *Pseudomonas fluorescens* ACB have also been employed in the regiodivergent oxidation of several bicycloketones.<sup>[38]</sup> The biotransformation of bicyclo-[3.2.0]hept-2-en-6-one led mainly to the unexpected lactone in low enantiomeric excess, whereas the expected lactone was achieved with 75 % *ee*. A similar trend was observed for the derivatives presenting a five-membered ring, for which the formation of the unexpected lactone with low enantiomeric excesses was favored, whereas the normal lactones presented much higher optical purities. In contrast, when oxidizing bicycloketones containing a six-membered ring, the major product was the normal lactone. In this case, the highest enantiomeric excesses were measured for the unfavored abnormal lactones. These examples again demonstrate the ability of BVMOs to tune regio- and/or enantioselectivity.

HAPMO has been also used in the regioselective transformation of fluorobenzaldehydes to fluorophenols,<sup>[39]</sup> compounds with applications as pharmaceuticals, agrochemicals and materials. The Baeyer–Villiger oxidation of the starting aldehydes leads to the corresponding esters, which are hydrolyzed to fluorophenols as main products, whereas only about 5% of fluorobenzoic acid (the other regioisomeric product) was observed. The use of the biocatalyst allows synthesis of the regioisomer (phenol ester) that is opposite to the one that is achieved by conventional chemical methods (benzoic acid); hence, this transformation can be considered as the biological equivalent to the Dakin reaction. The atypical outcome of this enzymatic oxidation is probably due to specific interactions of the benzaldehyde substrate with amino acids and flavin cofactor in the active site of HAPMO that favor the migration of the phenyl group.

BVMO as biocatalysts for the synthesis of  $\beta$ -amino acids

$\beta$ -Amino acids are of considerable industrial interest due to their application as building blocks for the preparation of  $\beta$ -peptides, alkaloids, terpenoids, and  $\beta$ -lactam antibiotics. Recently, a set of BVMOs from different bacterial origin have been employed as biocatalysts in the oxidation of linear aliphatic ketones possessing an amino group in the  $\beta$  position.<sup>[40]</sup> Whereas no reaction was observed for all the BVMOs tested when the amino group was unprotected, four of these enzymes [cyclodecanone monooxygenase (CDMO; EC 1.14.13.x)<sup>[41]</sup> from *Rhodococcus ruber* SC1, CHMO<sub>Arthor</sub>, CHMO<sub>Brachy</sub> and CHMO<sub>Xantho</sub>] were active on (±)-methyl (2-methyl-6-oxooctan-4-yl)carbamate **15**, leading to the formation of both possible regioisomeric lactones with excellent enantioselectivities. CDMO oxidizes the (+)-enantiomer of the starting ketones, generating the (+)-lactones, whereas the rest of the



**Scheme 5.** Enzymatic synthesis of β-amino-4-methyl-1-pentanol and *N*-protected-β-leucine by combining a BVMO-catalyzed oxidation with subsequent hydrolysis catalyzed by *Candida antarctica* lipase B. a) *E. coli* whole cells, BVMO; b) CAL-B, 50 mM phosphate, pH 7.5.<sup>[40]</sup>

BVMOs generate the (–)-enantiomer. After isolation and purification of both protected β-amino esters (16 and 17) and hydrolysis by *Candida antarctica* lipase B, *N*-protected-β-leucine (18) and *N*-protected β-amino-4-methyl-1-pentanol 19 (α-leucine precursor) was obtained (Scheme 5). Whereas only a restricted set of BVMOs and β-amino acid precursors have been explored, it is likely that with other BVMO types a much broader range of β-amino acids can be prepared by this novel BVMO-based process. This conceptionally new biocatalytic route to β-amino acids nicely illustrates the biocatalytic potential of BVMOs. The study has been recently extended by employing a collection of 16 BVMOs in the Baeyer–Villiger oxidation of different 4-amino-2-ketones and 5-amino-3-ketones,<sup>[42]</sup> being found excellent selectivities ( $E \geq 200$ )<sup>[43]</sup> in the resolution of aromatic and aliphatic 4-amino-2-ketones. The intermediate β-amino alkyl acetates underwent autohydrolysis, yielding the optically pure β-amino alcohols.

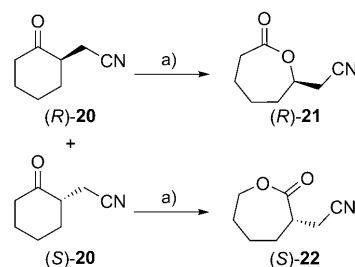
### 3. Kinetic and Dynamic Kinetic Resolutions Applied to BVMO-Catalyzed Processes

#### Kinetic resolutions of racemic ketones catalyzed by BVMOs

The resolution of racemic mixtures is nowadays a popular and important industrial approach for the synthesis of compounds in an optically active form. Whereas quite some examples have been described in which BVMOs were employed for the desymmetrization of prochiral compounds, the kinetic resolution of racemic ketones in order to obtain enantiopure ketones, esters or lactones had not been intensely studied until recently. Only some experiments have been developed in the kinetic resolution of substituted cycloalkanones.<sup>[18b,44]</sup> In the last few years several studies have appeared that demonstrate the use of BVMOs in kinetic resolutions. Recently, a set of cyanocyclohexanones have been oxidized by whole cells of *E. coli* expressing CHMO<sub>Acinetor</sub>.<sup>[45]</sup> Ketones possessing a cyano group in their structure are highly interesting in organic synthesis because the nitrile moiety can be transformed into several other groups, like amides, acids, or amines, providing access to a wide set of interesting compounds. Under the employed con-

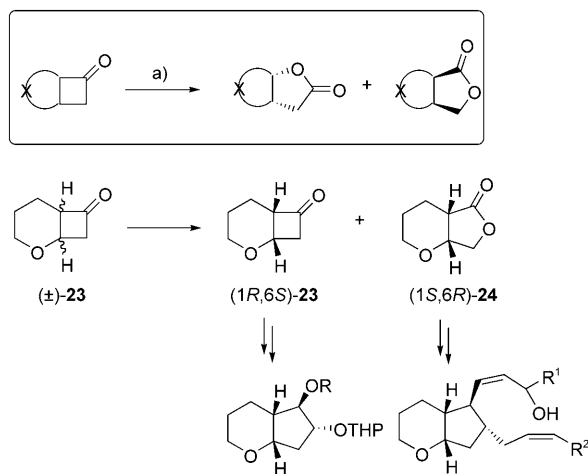
ditions, the nitrile group did not undergo any reaction, confirming the absence of nitrile hydratases or nitrilases in the employed *E. coli* hosts. The study showed that when the cyano group was directly attached to cyclohexanone, no reaction was observed (as expected for an enolizable carbonyl group). However, excellent results were obtained with the cyanoethyl derivative because it gave a highly enantioselective conversion ( $E \geq 200$ ). The conversion yielded the normal caprolactone of the *R* configuration, whereas it was also possible to recover the *S*-ketone. In contrast, the biooxidation of 2-cyanomethylcyclohexanone ( $\pm$ )-20 led to a regiodivergent parallel kinetic resolution in which both regioisomeric lactones are formed,<sup>[46]</sup> each one arising from one of the two enantiomers of the starting ketone, as shown in Scheme 6. The formation of the expected (*R*)-lactone 21 is slightly faster than the unexpected one with the *S* configuration, 22, but both products could be recovered as enantiopure compounds ( $ees \geq 99\%$ ).

*E. coli* cells expressing cyclopentadecanone monooxygenase from *Pseudomonas* sp. (CPDMO) have also recently been used in kinetic-resolution processes. The resolution of racemic 2-methylcyclopentadecanone occurs with low conversion and a very poor enantioselectivity, yielding the final (*S*)-lactone in 65% *ee*. Much better results were shown by this enzyme when oxidizing ( $\pm$ )-2-methylcyclohexanone. An excellent *E* value was achieved in the formation of enantiopure (*S*)-2-methylcaprolactone with 46% conversion. This enzyme led to low or moderate enantioselectivities when resolving substituted racemic cyclopentanones.<sup>[47]</sup>



**Scheme 6.** Regiodivergent parallel kinetic resolution of 2-cyanomethylcyclohexanone catalyzed by whole cells of CHMO from *Acinetobacter calcoaceticus*.<sup>[45]</sup> a) *E. coli* cells, CHMO<sub>Acinetor</sub>.

A BVMO from *M. tuberculosis* H37Rv has been expressed in *E. coli* and employed as biocatalyst in the kinetic resolution of fused bicyclic ketones (Scheme 7).<sup>[36]</sup> Interestingly, almost com-



**Scheme 7.** Kinetic resolution of fused bicyclic ketones catalyzed by whole cells of *E. coli* expressing a BVMO from *Mycobacterium tuberculosis* H37Rv.<sup>[46]</sup> a) BVMO.

plete conversions were observed after 48 h, affording the unexpected lactones with around 70% regioisomeric excess. Low enantiomeric ratios were measured for all the substrates. This enzyme was also used as biocatalyst in the selective oxidation of heterobicyclic fused ketones containing oxygen, yielding low or moderate enantioselectivities with the exception of racemic 2-oxabicyclo[4.2.0]octan-7-one **23**, which was oxidized with complete regioselectivity to the unexpected lactone 3-oxabicyclo[3.3.0]octan-2-one. Both (1*S*,6*R*)-(–)-lactone **24** and (1*R*,6*S*)-(+)-ketone **23** were obtained in enantiopure form after 50% conversion. This biotransformation is of high interest because the chiral starting ketone is a key intermediate in the synthesis of prostanoid synthons, and the lactones are interesting precursors for the synthesis of natural compounds presenting a tetrahydropyran core.

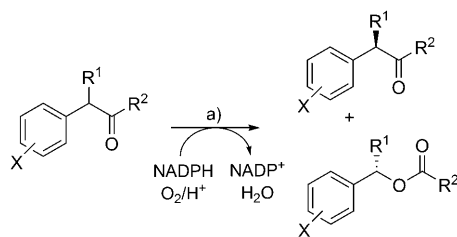
Whole cells of recombinant *E. coli* expressing the recently discovered BVMO from *Pseudomonas fluorescens* DSM 50106 were employed for the first time in the kinetic resolution of racemic aliphatic ketones. This biocatalyst has been previously identified as a BVMO with high specificity for aliphatic ketones.<sup>[48]</sup> It can be used to obtain octyl acetate, heptyl propionate, and hexyl butyrate and even longer alkyl chain esters. For a kinetic resolution, this BVMO was employed in the selective oxidation of a set of racemic 4-hydroxy-2-ketones, affording the (*S*)-hydroxyalkyl acetates with good enantioselectivities (*E* values around 50).<sup>[49]</sup> An acetyl migration of the final esters from the primary to the secondary hydroxy group was observed, yielding a 4:1 mixture of the monoacetylated 1,2-diols. Only small amounts of the other possible Baeyer–Villiger oxidation products were observed (<5%). Kinetic resolution of racemic hydroxyketones was then extended by employing a large set of substrates and by analyzing eleven additional BVMOs of various bacterial origin.<sup>[50]</sup> All of the tested enzymes oxidized

the *S* enantiomer of the starting material, yielding the (*R*)-hydroxyketones. In general, short and middle-chain ketones were preferred with a loss in enantioselectivity when increasing the alkyl chain of the starting ketones. Protection of the hydroxyl group before the biocatalyzed oxidation led to lower substrate acceptance of the CHMO-type enzymes. Again, the formed (*S*)-hydroxyalkyl esters undergo acyl migration to form the corresponding acetates of 1,2-diols in optically active form. Furthermore, the location of the carbonyl group in the substrate can influence the regioselectivity of the oxidation. Although methyl esters as atypical oxidation products were formed in low yields for 4-hydroxy-2-ketones (less than 10%), the use of 5-hydroxy-3-ketones led to more pronounced formation of the unexpected oxidation product.

Racemic benzylketones have also recently been tested as substrates of BVMOs in kinetic-resolution processes. Thus, *E. coli* cells expressing the BVMO from *P. fluorescens* DSM 50106 as well as CHMO<sub>Acinetor</sub> CPMO<sub>Coma</sub> and a BVMO from *Pseudomonas putida* KT2440<sup>[51]</sup> were used as oxidative biocatalysts in the resolution of racemic (±)-3-phenylbutan-2-one and (±)-3-phenylpentan-2-one to obtain the corresponding (*S*)-esters and (*R*)-ketones.<sup>[52]</sup> With exception of the kinetic resolution catalyzed by CPMO<sub>Coma</sub>, which occurred with high stereoselectivity, low to moderate *E* values were achieved. Much better results were obtained when employing the isolated BVMOs phenylacetone monooxygenase (PAMO; EC 1.14.13.92) from *Thermobifida fusca* and HAPMO.<sup>[53]</sup> These two enzymes, primarily active on aromatic substrates, were able to oxidize a set of short-alkyl-chain benzylketones with excellent enantioselectivities providing access to (*S*)-benzylesters and remaining (*R*)-benzylketones. For all the compounds tested, reactions were much faster and selective when employing PAMO. Some of the reaction parameters that can affect the biocatalytic properties were studied in the resolution of (±)-3-phenylpentan-2-one. This revealed that a higher pH (pH 9.5) resulted in higher productivity.<sup>[54]</sup> Concerning the temperature, it was shown that 40 °C was the optimum temperature for PAMO (this enzyme is even able to catalyze oxidations at 60 °C), whereas the maximum performance for HAPMO was found at 20 °C. Finally, substrate concentrations higher than 10 g L<sup>−1</sup> inactivated HAPMO, whereas PAMO is not affected to such an extent by the amount of substrate. This confirms the robustness of PAMO as biocatalyst, being derived from a thermophilic bacterium.<sup>[55]</sup>

This study was further extended to other benzylketones, including the recently cloned and overexpressed M446G mutant of PAMO (Scheme 8 and Table 2).<sup>[56]</sup> 3-Phenylbutan-2-ones with different substituents in the aromatic moiety were resolved by a selective oxidation processes, leading in all cases to the (*S*)-esters and the (*R*)-ketones. PAMO shows higher reactivity than its mutant and HAPMO, and the highest conversions were obtained for ketones bearing electron-withdrawing groups in the aromatic ring (3-trifluoromethyl and 4-nitro). The BVMO-catalyzed oxidation of long-alkyl-chain benzylketones was found to be a process with low conversion and poor *E* values. Only the M446G PAMO-catalyzed oxidation of (±)-4-phenylheptan-3-one and the HAPMO-based kinetic resolution of (±)-4-phenylhept-6-en-3-one progress with high enantioselectivities, albeit





**Scheme 8.** Isolated BVMO-catalyzed kinetic resolution of *rac*-benzylketones to obtain optically active (*R*)-benzylketones and (*S*)-benzylesters. a) Tris-HCl 50 mM, BVMO, G6P/G6PDH.

**Table 2.** Preparation of chiral benzylketones and benzylesters by kinetic resolution catalyzed by isolated BVMOs.

R <sub>1</sub>	R <sub>2</sub>	X	BVMO	t [h]	c [%] <sup>[a]</sup>	E <sup>[b]</sup>
Me	Me	H	PAMO <sup>[c]</sup>	1	27	188
Me	Me	3-Me	HAPMO <sup>[d]</sup>	2	20	126
Me	Me	3-CF <sub>3</sub>	HAPMO <sup>[d]</sup>	2	39	112
Me	Me	4-NO <sub>2</sub>	M446G <sup>[d]</sup>	1	50	121
Me	Et	H	PAMO <sup>[c]</sup>	1	19	> 200
Et	Me	H	PAMO <sup>[c]</sup>	1	32	> 200
Pr	Et	H	HAPMO <sup>[d]</sup>	4	23	66
Allyl	Et	H	HAPMO <sup>[d]</sup>	4	5	104

[a] Determined by GC or HPLC. [b] See ref. [43]. [c] See ref. [55]. [d] See ref. [57].

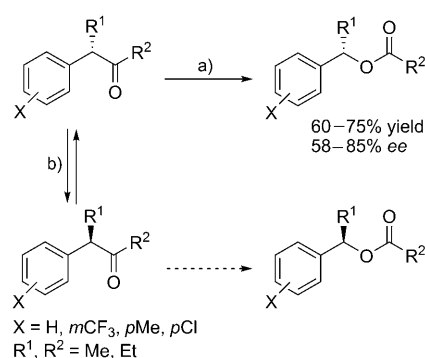
with low conversions. The results shown that by proper selection of a BVMO, moderate to good *E* values can be achieved for kinetic resolution of aromatic ketones.<sup>[57]</sup>

#### BVMOs in dynamic kinetic resolution processes

The main drawback kinetic-resolutions processes present is that the theoretical yield for both substrate and product is limited to 50%. Strategies to increase the yield are therefore of great importance. By combining an in situ racemization of substrate with an enzymatic resolution, a dynamic kinetic resolution (DKR) can be achieved. This allows the transformation of both enantiomers of the starting material into a single enantiomeric product in 100% theoretical yield. For obtaining an efficient DKR process, certain requirements must be fulfilled, such as the irreversibility of the kinetic resolution, the absence of product racemization and the need for good enantioselectivity in the catalyzed resolution step. Numerous successful examples of DKR methods have been described recently.<sup>[58]</sup> Some of them involve only conventional chemical methods, but in others chemical and biocatalytic processes are combined. In the latter case, the biocatalyst can act as racemization agent or contribute to the kinetic resolution. Reports on BVMO-based DKRs are scarce but a few examples were recently reported.

The Alphand/Furstoss group has described the first example of dynamic kinetic resolution catalyzed by BVMOs.<sup>[59]</sup> *E. coli* cells expressing CHMO<sub>Acinetobacter</sub> were employed in the selective oxidation of racemic 2-benzyloxymethylcyclopentanone. This substrate, bearing an acidic hydrogen in position  $\alpha$  to the carbonyl moiety, spontaneously racemizes via keto–enol tautomerization

leading to (*R*)-6-benzyloxymethyltetrahydropyran-2-one with 75% yield and 98% enantiomeric excess. However, this methodology suffered from certain important disadvantages: 1) the need for working at high pH, at which a loss in enzymatic activity for particular biocatalysts is observed; 2) the substrate concentration had to be low and 3) the protocol is only applicable to acidified  $\alpha$ -centers. An improved DKR process has been developed by combining the whole-cell-catalyzed Baeyer–Villiger oxidation with an in situ racemization, catalyzed by a weak anion-exchange resin. Thus, enzymatic oxidations can be performed at neutral pH, optimal for CHMO<sub>Acinetobacter</sub> activity, and substrate concentration can be relatively high. With this method, (*R*)-6-benzyloxymethyltetrahydropyran-2-one can be obtained in 84% yield and 97% enantiomeric excess.<sup>[60]</sup> Recently, the preparation of a set of optically active benzylesters with high conversions and enantiomeric excesses has been described by performing the DKR of racemic benzylketones employing isolated HAPMO.<sup>[61]</sup> The initial studies were performed with ( $\pm$ )-3-phenylbutan-2-one. This ketone maintains its optical purity at relatively high pH, whereas it readily racemizes in the presence of anion-exchange resins. Thus, the oxidation of the substrate was carried out in the presence of different anion-exchange resins. Resins displaying a strong character (quaternary ammonium functional group) led to a fast substrate racemization, but also were found to inactivate the biocatalyst. Worse racemization but much better biocatalytic results were obtained with weak resins with a tertiary amine functional group. (*S*)-Phenylethyl acetate could be obtained with 84% enantiomeric excess and 86% conversion in the optimal reaction conditions. This methodology was extended to the preparation of other (*S*)-benzylesters with moderate to good conversions and enantiomeric excesses depending on the substrate structure, as indicated in Scheme 9.



**Scheme 9.** DKR of racemic benzylketones by combining the HAPMO-catalyzed Baeyer–Villiger oxidation and racemization by an anion-exchange resin.<sup>[61]</sup> a) HAPMO/Tris-HCl, pH 10, G6P/G6PDH/NADPH, 25 °C/250 rpm; b) Dowex MWA1.

## 4. Use of Baeyer–Villiger Monooxygenases for Other Oxidative Processes

### BVMO-catalyzed sulfoxidation reactions

As mentioned above, BVMOs catalyze not only the Baeyer–Villiger reaction, but also various other oxidation reactions.

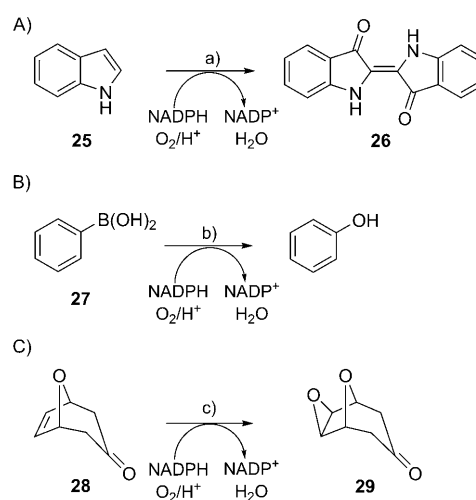
Whereas all BVMOs discovered so far are merely meant to catalyze a Baeyer–Villiger reaction in nature, their ability to catalyze for example, enantioselective sulfoxidations makes them highly attractive for synthetic applications. The BVMO-catalyzed oxidation of prochiral sulfides is an interesting process that leads to the formation of chiral sulfoxides,<sup>[62]</sup> compounds intensely employed in organic chemistry as auxiliaries in asymmetric synthesis and chiral ligands in enantioselective catalysis. Chiral sulfoxides have also been used as key intermediates in the preparation of biologically active compounds or possess this activity by themselves. The oxidation of organic sulfides was first studied by the Walsh group by using CHMO<sub>Acineto</sub> as a biocatalyst,<sup>[63]</sup> and afterwards this enzyme has been employed in the preparation of a wide set of chiral sulfoxides, usually with high selectivities.<sup>[32,64]</sup> Purified PAMO has been recently employed as a chiral catalyst for the synthesis of various chiral aromatic sulfoxides. This enzyme was able to catalyze the enzymatic sulfoxidation of alkyl phenyl sulfides with moderate enantioselectivity. Better results were achieved in the sulfoxidation of alkyl benzyl sulfides, especially for those possessing a methyl or ethyl alkyl chain (*ees* ≥ 94%).<sup>[65]</sup> For some of the sulfoxides, a change in their optical purity was observed as a function of time, indicating that this BVMO was also able to catalyze the enantioselective kinetic resolution of racemic sulfoxides, oxidizing preferably one enantiomer of the racemic mixture to the achiral sulfone. High *E* values were obtained for benzyl methyl and benzyl ethyl sulfoxides, indicating that the higher optical purities achieved by conversion of the corresponding sulfides are due to the asymmetric oxidation of the sulfide combined with the kinetic resolution of the formed sulfoxide to the sulfone. Purified HAPMO has also been used in the preparation of chiral aromatic sulfoxides, presenting a different behavior to that observed for PAMO.<sup>[66]</sup> This enzyme showed a high preference for the oxidation of alkyl phenyl sulfides, yielding the (*S*)-alkyl phenyl sulfoxides with good to excellent enantiomeric excesses. HAPMO was also shown to be able to perform enantioselective sulfoxidations of bulky aromatic sulfides: for example, it oxidizes methyl 2-naphthyl sulfide with a high selectivity (*ee* = 95%).

Chiral  $\beta$ -hydroxysulfoxides are of special interest as chiral auxiliaries and as building blocks for the synthesis of compounds such as benzoxathiepinines, allylic alcohols, or macrocyclics. Recently, a biocatalyzed procedure for the synthesis of these compounds has been described by oxidizing the corresponding  $\beta$ -hydroxysulfides by using isolated CHMO<sub>Acineto</sub>.<sup>[67]</sup> Sulfoxidation of *trans*-2-(phenylsulfinyl)cyclohexan-1-ol occurred with excellent regio- and enantioselectivity for the preparation of the (1*S*,2*S*)- $\beta$ -hydroxysulfoxide.

### Other BVMO-catalyzed oxidations

It has been described that BVMOs are able to perform a set of alternative oxidation reactions, such as the biotransformation of selenides to selenoxides,<sup>[68]</sup> boronic acids to phenols,<sup>[68]</sup> tertiary amines to *N*-oxides<sup>[69]</sup> and even the epoxidation of double bonds to the corresponding epoxides.<sup>[70]</sup>

CHMO<sub>Acineto</sub> has been recently employed in the oxidation of secondary amines under mild reaction conditions, yielding the corresponding nitrones as the final products.<sup>[71]</sup> These compounds are highly versatile synthetic intermediates, especially as 1,3-dipoles, and they possess pharmacological activity. The BVMO-catalyzed oxidation occurred through a double oxidation, starting with oxidizing the amine followed by oxidation of the hydroxylamine intermediate which led to two regioisomeric nitrones. As expected, the use of bulkier secondary amines led to lower conversions. The M446G PAMO mutant has also been able to convert indole (**25**), a secondary amine, into indigo blue (**26**) during cultivation (Scheme 10A).<sup>[56]</sup> Whereas the exact mechanism of this oxidation reaction is ob-



**Scheme 10.** Alternative oxidation reactions performed by BVMOs. A) M446G PAMO biooxidation of indole to indigo blue,<sup>[56]</sup> B) Biocatalyzed oxidation of phenylboronic acid to phenol,<sup>[65,66]</sup> and C) Epoxidation of 7-oxabicyclo[2.2.1]hept-5-en-2-one catalyzed by CHMO<sub>Xantho</sub>.<sup>[29]</sup> a) *E. coli* cells, M446G PAMO; b) PAMO or HAPMO; c) CHMO<sub>Xantho</sub>.

scure, it is thought to be initiated by *N*-oxidation of the endogenous indole generated from tryptophan by tryptophanase in *E. coli*. This is in line with the fact that the enzyme is also able to oxidize for example, *N*-methylbenzylamine. The conversion of indole into indigo blue could also be performed by using isolated enzyme. Whereas this was the first study in which a BVMO was shown to be able to produce indigo blue, recently other BVMOs were identified from a metagenomic library that are also able to convert indole into indigo blue.<sup>[72]</sup>

Another documented activity of BVMOs is the oxidation of boron-containing compounds. In a recent study it has been shown that such reaction is also feasible with PAMO and HAPMO because they are able to oxidize phenylboronic acid (**27**) into phenol (Scheme 10B).<sup>[65,66]</sup> For a long time the epoxidation of vinyl-phosphonates by CHMO<sub>Acineto</sub> was the only described BVMO-mediated epoxidation.<sup>[70]</sup> Interestingly, recently it was discovered that the oxidation of the bicyclic ketone 7-oxabicyclo[2.2.1]hept-5-en-2-one (**28**) by whole cells of the BVMO from *Xanthobacter* sp. did not lead to the Baeyer–Villiger oxidation product. Instead, formation of the epoxide **29**

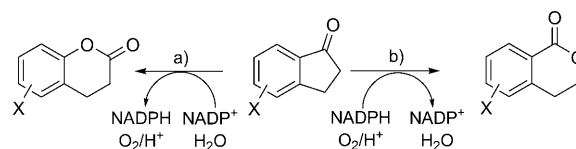
from oxidation of the double bond was observed. This exceptional behavior for a BVMO can be rationalized by the electron-rich character of the double bond in combination with a particular secondary interaction by the oxygen bridge, which can lead to an electrophilic oxygenation to form the corresponding epoxide (Scheme 10C).<sup>[29]</sup>

## 5. Special Modes of Applying Baeyer–Villiger Monooxygenases

### BVMO-catalyzed oxidations in presence of organic cosolvents

In nature, biocatalysts typically act in an aqueous medium. However, it has been established since more than 20 years that enzymes can also be active in reaction media containing organic solvents.<sup>[73]</sup> Biocatalytic reactions in organic media present some advantages compared to the use of aqueous solutions, for example, 1) higher solubility of hydrophobic substrates, 2) enabling reactions not accessible in water, 3) prevention of unwanted side reactions, 4) facilitating recovery and recycling of enzymes, and 5) solvents have been shown to improve chemo-, regio- and/or enantioselectivity. Unfortunately, the use of organic solvents also often causes drawbacks, such as the lower enzymatic activity or the limitation of mass transfer in the case of biphasic systems. Hydrolytic enzymes have been widely used in organic reaction media, whereas only relatively few examples in which oxidoreductases were employed in nonaqueous systems have been described.<sup>[74]</sup>

In the last few years several studies have focused on the use of BVMOs in the presence of organic solvents. It has been found that the Baeyer–Villiger oxidation of benzofused ketones proceeded with higher conversions by employing different organic cosolvents in a 5% (v/v) concentration in the reactions catalyzed by isolated PAMO, its M446G mutant, and HAPMO.<sup>[75]</sup> Tetralones were bad substrates for the three enzymes in buffer. However, the addition of 5% of hydrophobic solvents, such as toluene or 2-octanol, allowed to convert 1-tetralone into 4,5-dihydro-1-benzoxepin-2(3H)-one. This clearly demonstrates the benefits of applying organic (co)solvents for biocatalysis. When conversion of 2-indanone was studied, addition of 5% of cosolvents was found to improve the conversion, leading to a 90% conversion when using 1,4-dioxane. Considering oxidation of 1-indanone and derivatives substituted in the aromatic moiety, also better conversions could be achieved by adding organic solvents. HAPMO leads to the formation of the “expected” lactones with improved conversions when adding hydrophobic solvents (hexane, toluene, or octan-2-ol), whereas M446G PAMO is able to form the “unexpected” lactone with the best results in 5% of hydrophilic solvents (methanol, 1,4-dioxane, or isopropanol). Thus, by selecting the biocatalyst and reaction media properly, both regioisomeric lactones could be obtained from a set of 1-indanones with excellent yields, as indicated in Scheme 11. It was also shown that HAPMO is still active in mixtures with 50% hexane whereas M446G PAMO can tolerate 30% methanol.

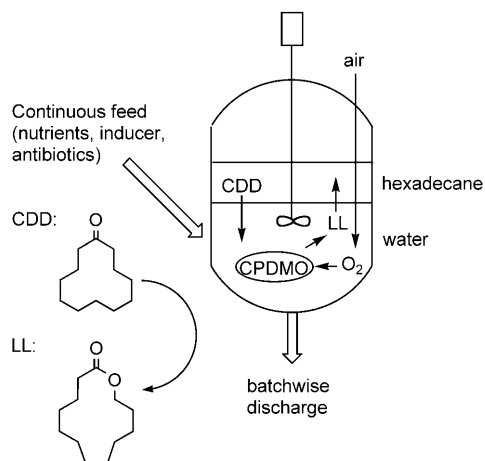


**Scheme 11.** Regiodivergent Baeyer–Villiger oxidation of substituted 1-indanones by employing the isolated BVMOs from *Pseudomonas fluorescens* ACB and the M446G mutant from *Thermobifida fusca*.<sup>[75]</sup> a) HAPMO / 5% hexane; b) M446G PAMO / 5% methanol.

The use of biphasic reaction media can also serve to stabilize BVMOs under reaction conditions with high concentrations of substrate. A recently engineered PAMO mutant “P3”<sup>[76]</sup> suffered a drastic deactivation when the substrate concentration was increased to more than 1 g L<sup>−1</sup> in the whole-cell oxidation of different ketones. To overcome this inhibition, a second organic phase was employed as a substrate reservoir and product sink, resulting in full conversions in a mixture of buffer/dioctylphthalate 1:1 at ketone levels of 3 g L<sup>−1</sup>. Reactions were also carried out by employing the purified enzyme coupled to the alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* as a cofactor regeneration system. The presence of detergents such as Tween 20 had a positive effect on PAMO mutant stability when working in biphasic media with 50% organic cosolvents. The use of cyclohexane and *tert*-butyl methyl ether had only a moderate influence on the biocatalyst, whereas other cosolvents led to a fast enzymatic deactivation. Thus, a 200 mL biotransformation of 5 g L<sup>−1</sup> of 4-phenylcyclohexanone using *tert*-butyl methyl ether and aqueous buffer as reaction medium catalyzed by PAMO-P3 has been described.<sup>[77]</sup>

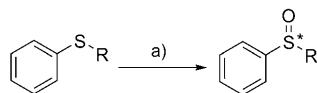
Recombinant *E. coli* whole cells containing CPDMO have also been used in the oxidation of cyclododecanone to lauryl lactone. This was done out in a two-phase partitioning bioreactor (TPPB), consisting of an organic and an aqueous phase that contains the biocatalyst and the growth media. The biotransformation performed in a batch process by using hexadecane/water as reaction media led to a maximum lauryl lactone production of 2.4 g L<sup>−1</sup> after 10 h. These results were improved by using a variation of the TPPB system, the two-phase semicontinuous reactor (TPSCR, Figure 1) in which the cells are grown in the aqueous phase by feeding nutrients.<sup>[78]</sup> The organic phase serves as a carrier of the reactant and an extraction medium for the in situ removal of product. Mixing and aeration of the system were interrupted for 20 min to allow the separation of the layers, which discharged part of the aqueous phase containing aged cells, metabolites, and debris of dying cells while the organic phase was maintained. When the used cells are periodically discharged (ten times) over a period of 132 h, a plateau in lauryl lactone (99 mM) was observed after 72 h, demonstrating that the TPSCR system extended the productivity over a longer period of time compared to the batch mode. A further increase in productivity was achieved by increasing the substrate concentration to 840 mM, which enabled production of 11 g L<sup>−1</sup> of lauryl lactone after 72 h.

Recently it was shown that the use of unconventional media can also improve or even inverse the enantioselective behavior



**Figure 1.** CPDMO-biocatalyzed production of lauryl lactone (LL) from cyclo-dodecanone by employing a two-phase semicontinuous reactor (TPSCR).<sup>[78]</sup>

of BVMOs.<sup>[79]</sup> The first example of this was shown in the sulfoxidation of alkyl aryl sulfides when employing purified HAPMO, PAMO and the ethionamide monooxygenase from *Mycobacterium tuberculosis* (EtaA).<sup>[80]</sup> Although the use of organic solvents (30%, v/v) was found to diminish enzymatic activity to some extent, this negative effect was counterbalanced by the increase of the solubility of the starting sulfides in the unconventional media. Moreover, when PAMO or EtaA were employed as biocatalysts in the oxidation of thioanisole in 30% methanol or ethanol, a significant increase in enantioselectivity was observed, enabling the synthesis of methyl phenyl sulfoxide with high optical purities (Scheme 12, Table 3). Moreover, the use of



**Scheme 12.** BVMO-catalyzed sulfoxidation of thioanisole and analogues by using unconventional reaction media aqueous buffer/organic cosolvent.<sup>[79]</sup> a) PAMO or EtaA, Tris-HCl, pH 9.0/cosolvent, G6P/G6PDH/NADPH, 250 rpm.

methanol inverted the enantiopreference of PAMO when oxidizing ethyl phenyl sulfide or phenyl propyl sulfide, yielding the (*R*)-sulfoxides in excess instead of the (*S*)-sulfoxides. The same behavior was also observed when adding 30% *tert*-butyl methyl ether to the EtaA-catalyzed oxidation of thioanisole. The effect of methanol in the PAMO-catalyzed oxidation was analyzed in more detail. With only 1% of this cosolvent, it was possible to increase the *ee* of the final sulfoxide from 43 to 68%. A further increase in the methanol concentration led to (*R*)-methyl phenyl sulfoxide in 89% *ee*. The substrate concentration did not alter the enantioselectivity of PAMO, and the highest reaction rate (expressed as grams of substrate transformed per L per h) was achieved at 20 g L<sup>-1</sup> h<sup>-1</sup>.

The performance of PAMO in the presence of organic cosolvents was also studied for the Baeyer–Villiger oxidation of a

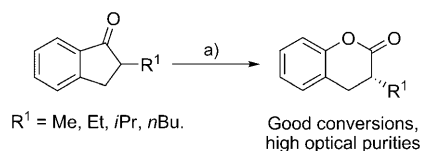
**Table 3.** Effect of organic cosolvents in the biocatalytic properties of PAMO and EtaA when oxidizing prochiral sulfides.

R	BVMO	Cosolvent [%]	c [%] <sup>[b]</sup>	ee [%] <sup>[c]</sup>	Config.
Me	PAMO	none	94	44	<i>R</i>
Me	PAMO	MeOH (1 %)	83	68	<i>R</i>
Me	PAMO	MeOH (30 %)	32	89	<i>R</i>
Me	PAMO	EtOH (30 %)	9	48	<i>R</i>
Et	PAMO	none	79	33	<i>S</i>
Et	PAMO	MeOH (30 %)	26	87	<i>R</i>
Pr	PAMO	none	56	19	<i>S</i>
Pr	PAMO	MeOH (30 %)	9	56	<i>R</i>
Me	EtaA	none	43	33	<i>S</i>
Me	EtaA	MeOH (30 %)	10	84	<i>S</i>
Me	EtaA	<i>t</i> BuOMe (30 %)	11	26	<i>R</i>

[a] Yield after purification by column chromatography. [b] Determined by GC. [c] Determined by HPLC.

set of racemic 3-phenylbutan-2-ones bearing different substituents in the aromatic ring.<sup>[81]</sup> Again, methanol triggered an improvement in the enantioselectivity of the kinetic resolution of several 3-phenylbutan-2-ones. The best conditions in terms of activity and *E* value were in 10–30% methanol. In the same study, beneficial effects of organic cosolvents in the PAMO-selective oxidation of  $\alpha$ -acetylphenylacetone to (*R*)-acetoxyphenylacetone were found. No reaction was observed in buffer with this ketone. However, the use of mixtures of aqueous buffer with 10% of water-immiscible solvents led to effective conversion. By using 10% EtOAc, it was possible to obtain a 56% yield of enantiopure (*R*)-cyanoester after 72 h. Thus, because the starting material easily racemizes under these conditions, an efficient DKR was performed by simply modifying the reaction media.

The enzymatic Baeyer–Villiger oxidation of 2-alkyl-1-indanones by using isolated M446G PAMO led to optically active 3-alkyl-3,4-dihydroisocoumarins, which represent interesting intermediates in the preparation of biologically active compounds. The use of relatively high pH and temperature allowed the racemization of the starting material and resulted in an effective DKR, leading to formation of the “unexpected” lactones, as shown in Scheme 13.<sup>[82]</sup> Optimization of the reaction conditions by exploring a set of organic solvents showed that oxidations performed with 5% methanol led to the highest conversions. However, the presence of this alcohol led to a drop in the selectivities, whereas the addition of 5% hexane al-



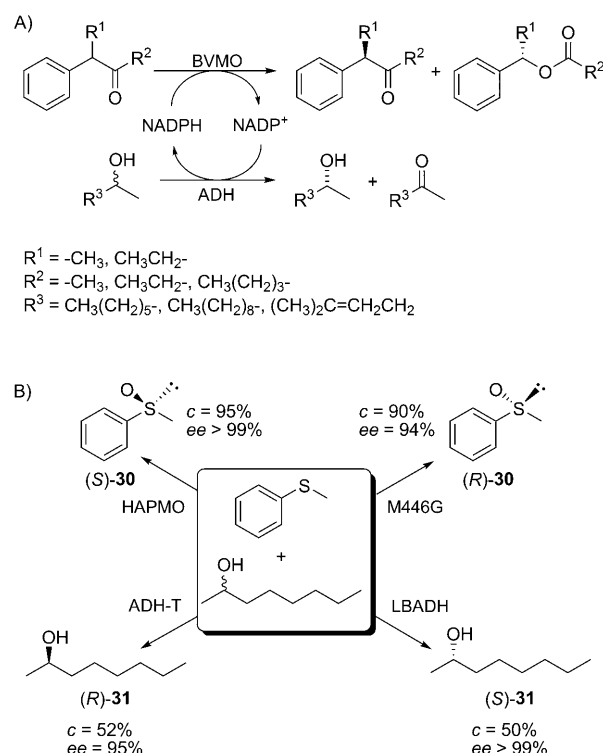
**Scheme 13.** Synthesis of 3-alkyl-3,4-dihydroisocoumarins by dynamic kinetic resolution employing M446G PAMO in unconventional reaction media.<sup>[83]</sup> a) M446G, PAMO, Tris-HCl 50 mM, pH 10, 5% *n*-hexane or MeOH, G6P/G6PDH/NADPH, 40 °C/250 rpm.



lowed the chiral benzofused lactones to be recovered in the highest optical purities; this demonstrates the beneficial effect of organic cosolvents in some BVMO-catalyzed reactions. Oxidations were more effective for those substrates with short alkyl chains, leading to 3-methyl- and 3-ethyl-3,4-dihydroisocoumarins with conversion higher than 80%, whereas the presence of longer chains produce a slight loss in conversions. All the final products can be obtained with high to excellent optical purities. Biooxidations were scaled up to 250 mg to obtain the final 3-alkyl-3,4-dihydroisocoumarins, which represent interesting bioactive molecules.

### Development of parallel interconnected kinetic asymmetric transformations by using BVMOs

In Nature a huge number of chemically interconnected processes concurrently take place, and the products are shared by different metabolic routes, forming a complex and effective metabolic network. In an attempt to mimic such efficient natural processes, concurrent catalytic concepts are being developed.<sup>[83,84]</sup> Recently, a methodology has been developed, described as parallel interconnected kinetic asymmetric transformation (PIKAT), which allows the concurrent preparation of enantioenriched derivatives in a parallel way, with the advantages of minimizing the quantity of reagents employed and maximizing the *redox economy* of the process. In such an approach, two asymmetric transformations were coupled: 1) through two kinetic resolutions or 2) through a kinetic resolution and a desymmetrization reaction. The PIKAT systems were constructed by coupling of two redox biocatalysts (an isolated ADH and an isolated BVMO) and by using a catalytic amount of nicotinamide coenzyme. The coenzyme acts as connector between the concurrent reactions. In a first approach, isolated BVMOs (PAMO, its M446G mutant or HAPMO) catalyzed the enzymatic kinetic resolution of a racemic benzylketone while converting NADPH to NADP<sup>+</sup>. This oxidized coenzyme was then internally recycled in a *one-pot* process by the selective oxidation of a racemic secondary alcohol by using the ADHs from *Thermoanaerobacter* sp (ADH-T) or from *Lactobacillus brevis* (LBADH). As final reaction products, chiral *sec*-alcohols, benzylketones, and benzylesters were obtained (Scheme 14A).<sup>[85]</sup> The reaction parameters were optimized to achieve both kinetic-resolution processes with excellent enantioselectivities. This concept was then extended to the desymmetrization of a set of prochiral sulfides through BVMO-catalyzed sulfoxidation coupled with the ADH-catalyzed kinetic resolution of different racemic secondary alcohols.<sup>[86]</sup> By simply changing the biocatalysts employed, the selectivity of the system can be tuned. By using this methodology, a set of chiral sulfoxides and chiral alcohols could be obtained with high optical purities. One PIKAT example is given in Scheme 14B, showing that all enantiomers of methyl phenyl sulfoxide (**30**) and of octan-2-ol (**31**) can be obtained with excellent conversions and enantiomeric excesses by selecting the right biocatalysts for the PIKAT system.

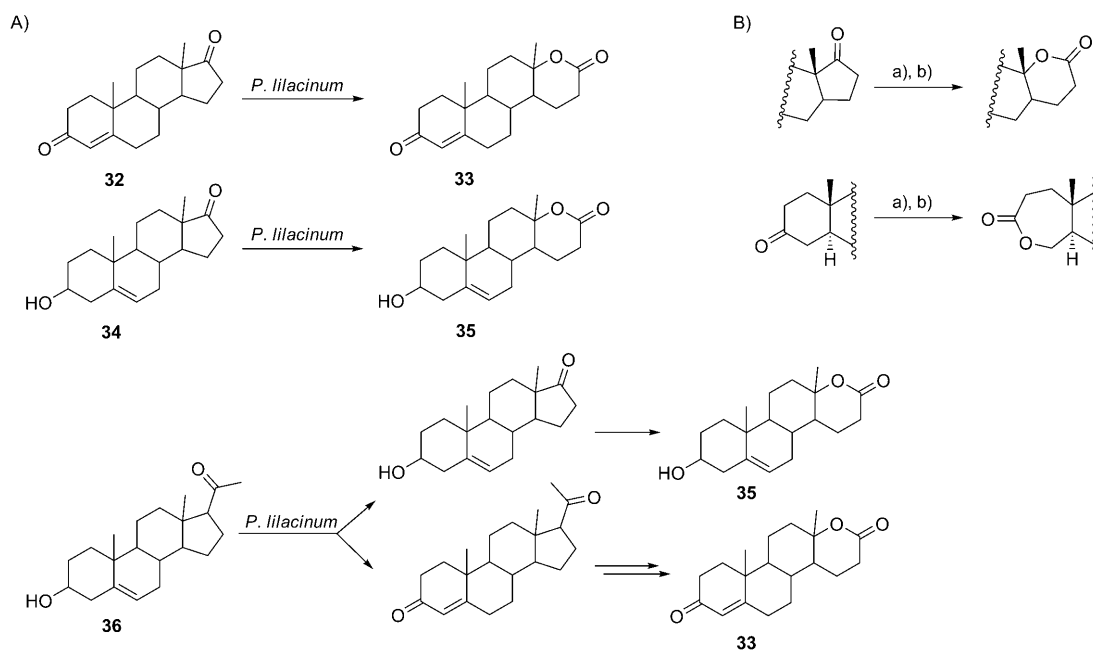


**Scheme 14.** A) Parallel interconnected kinetic asymmetric transformation of racemic benzylketones and racemic *sec*-alcohols by using BVMOs and ADHs; and B) Enzymatic synthesis of all enantiomers of methyl phenyl sulfoxide and *rac*-2-octanol catalyzed by HAPMO or M446G PAMO as BVMOs and the alcohol dehydrogenases ADH-T or LBADH.<sup>[85]</sup>

## 6. Applications of BVMOs in the Synthesis of Biologically Active Compounds

The majority of the above-described oxidations were performed with the goal of achieving the synthesis of optically active building blocks for the preparation of high value-added compounds. In addition, BVMOs have been utilized for the direct synthesis of biologically active compounds. Below, some BVMO-mediated conversions that yield biologically active products are described.

Steroids represent an important class of natural products with varying pharmacological properties, such as anticancer, antiandrogenic, and antihypercholesterolemic activity. Minor modifications in their structure can affect their biological activity. Consequently, numerous examples of new bioactive compounds have been described by subtle steroid modifications, in most of cases employing microorganisms. It has been shown that strains of the genus *Penicillium* can perform steroid transformations by means of reductions, hydroxylations, or Baeyer–Villiger oxidations (Scheme 15A).<sup>[87]</sup> The BVMO activity from *Penicillium lilacinum* AM111 is able to carry out D-ring oxidation and degradation of steroids. Thus, dehydroepiandrosterone (**32**) was oxidized to 3β-hydroxy-17α-oxa-Δ-homo-androst-5-en-17-one (**33**) whereas androstenedione (**34**) was converted into testololactone (**35**) in high yield. Baeyer–Villiger oxidation of pregnenolone (**36**) yielded a mixture of compounds,



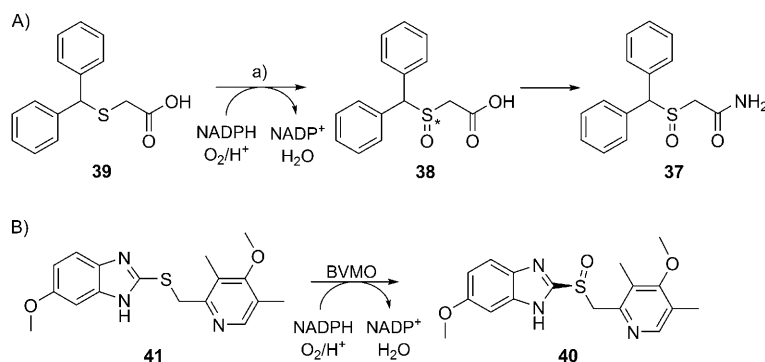
**Scheme 15.** Enzymatic Baeyer-Villiger oxidation of steroids catalyzed by BVMOs: A) Employing whole cells of *P. lilacinum* AM111,<sup>[87]</sup> and B) Isolated CPDMO-biocatalyzed oxidation of 17-keto- and 3-ketosteroids.<sup>[88]</sup> For this latter enzyme, Baeyer-Villiger reaction led to the formation of the 3-one-4-oxa-4a-homolactones, a regioisomeric product to the one obtained by employing peracids. a) CPDMO; b) G6P/G6PDH, NADPH.

with 3 $\beta$ -hydroxy-17 $\alpha$ -oxa- $\beta$ -homo-androst-5-en-17-one (**33**) as the major product and testolactone (**35**). This demonstrates that the use of microbial isolates can be useful for selective Baeyer-Villiger oxidations.

Only in a few cases has the use of isolated enzymes, such as cytochrome P450s or BVMOs, been explored within steroid transformations. Recently, isolated CPDMO from *Pseudomonas* sp. has been screened for the biooxidation of a large number of steroids.<sup>[88]</sup> Whereas this BVMO is involved in catabolism of cyclopentadecanone in nature, it was found that it also accepts steroids as substrates, as indicated in Scheme 15B. Thus, the oxidation of 17-ketosteroids was achieved albeit with low yields, yielding the corresponding normal lactones. The chemical Baeyer-Villiger oxidation of steroids bearing the carbonyl group in the A-ring (3-ketosteroids) employing peracids led to the formation of the 3-oxa-4-one-4a-homo products. The peculiar regioselectivity of this oxidation can be explained by the attack of the peracid to the less-hindered carbon due to the chair conformation of the Criegee intermediate. Biocatalyzed Baeyer-Villiger oxidation with CPDMO afforded the preparation of the opposite regioisomeric 3-one-4-oxa-4a-homolactones for the first time, but with low to moderate yields.

A BVMO-based methodology has also been employed for the preparation of the pharmaceutical (–)-modafinil (**37**), a drug

employed for the treatment of narcolepsy and other sleep disorders. Recently, the preparation of its key intermediate (–)-(2-benzydrylsulfinyl)acetic acid (**38**) has been described by employing isolated PAMO, which performs an enantioselective sulfoxidation of prochiral (–)-(2-benzydrylthio)acetic acid (**39**; Scheme 16A).<sup>[89]</sup> The final product could be obtained with complete conversion and good enantiomeric excess (85% ee). The optical purity of the modafinil precursor can be slightly increased by the addition of 10% methanol or isopropanol to the reaction medium. Another promising example of BVMO-mediated synthesis of a pharmaceutical is the use of an engineered BVMO for preparing esomeprazole, a proton-pump inhibitor (PPI) and multibillion dollar drug. Esomeprazole (**40**), a substituted pyridylmethylsulfinyl benzimidazole, is widely used for the treatment of acid-related diseases. Recently, a BVMO has been optimized by directed evolution to effectively per-

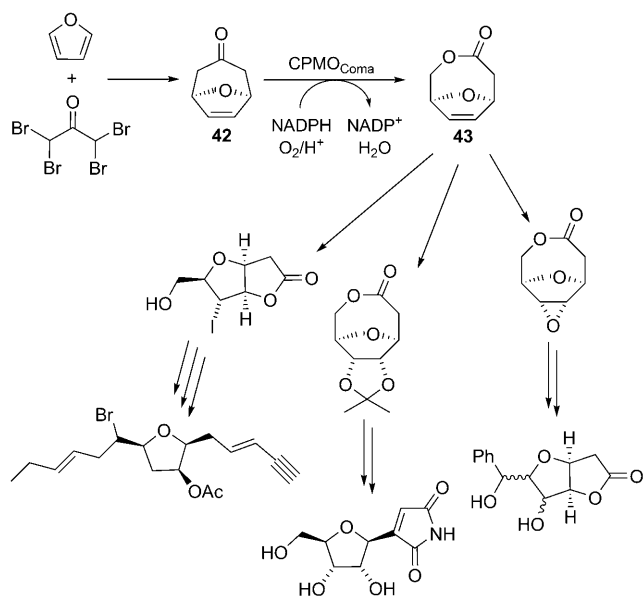


**Scheme 16.** A) Chemoenzymatic synthesis of (–)-modafinil employing as key step a PAMO-catalyzed sulfoxidation,<sup>[89]</sup> B) Biocatalyzed preparation of esomeprazole by using an evolved Baeyer-Villiger monooxygenase.<sup>[90]</sup> a) PAMO/buffer, G6P/G6PDH.

form the enantioselective sulfoxidation of omeprazole (**41**), leading to esomeprazole (Scheme 16B).<sup>[90]</sup>

Biocatalytic reactions are receiving increasing interest when these green chemistry methodologies can be integrated in sustainable strategies. Thus, a combined photochemical and biocatalytic methodology has been employed for the synthesis of bicyclo[4.2.0]octanes, a structural moiety that is widely spread in natural products and that is difficult to prepare by conventional chemical methodologies. The Cu-catalyzed [2 + 2] photocycloaddition of the *O*-*tert*-butyldimethylsilyl-protected 1,3-divinyl-2-cyclopentanol proceeded smoothly with high *endo* diastereoselectivity. Cleavage of the protecting group and oxidation of the resulting alcohol led to the bridge ketone, which allowed for several cleavage protocols. The microbial Baeyer–Villiger oxidation was found to be the most appealing approach in order to achieve good conversions.<sup>[91]</sup> The formation of enantiocomplementary lactones was found to depend on the enzyme used. By selecting the proper BVMO it is possible to get access to antipodal lactones with good to excellent enantiomeric excess. The final molecule, bicyclo[4.2.0]octane, was obtained after lactone hydrolysis.

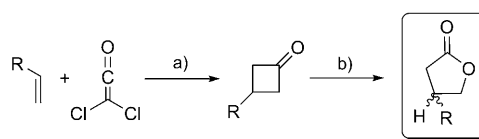
The preparation of the oxabicycloketone by a sonochemical [4 + 3] cycloaddition of furan and tetrabromoacetone with subsequent reductive dehalogenation afforded the interesting prochiral compound **42** in a single operation. This ketone was selectively oxidized by CPMO<sub>Coma</sub> leading to the chiral (+)-(1*S*,6*S*)-lactone **43** with high optical purity in a desymmetrization that introduces two stereogenic centers into the molecule, as indicated in Scheme 17.<sup>[92]</sup> After optimizing the fermentation conditions, an isolated yield of 70% can be achieved. The final product, presenting high functional diversity and structural rigidity, has been employed as starting material for the preparation of various natural products containing a tetrahydrofuran structural motif. Thus, the total syntheses of the antibiotic (+)-



**Scheme 17.** Chemoenzymatic synthesis of tetrahydrofuran-based compounds employing as key step a Baeyer–Villiger oxidation catalyzed by CPMO<sub>Coma</sub>.<sup>[92]</sup>

showdomycin (confirming the absolute configuration of the bio-oxygenation product) and the metabolite *trans*-kumausyne were described, as well as the formation of precursors of the cytotoxic metabolite goniofuranone and analogues. It is interesting to note that CHMO<sub>Xantho</sub> is also able to convert the carbocyclic analogue of compound **42** in >99% *ee*, which, in principal, allows access into the corresponding carbocyclic compound series.<sup>[29]</sup>

The BVMO-catalyzed oxidation of prochiral cyclobutanones provides access to chiral butyrolactones, a highly versatile platform to synthesize natural products and bioactive molecules with great structural diversity, like GABA receptor inhibitors,  $\beta$ -amino acids, lignans, or antagonistic analgesics. Several ketone precursors, prepared by a straightforward [2+2] cycloaddition, were oxidized by *E. coli* cells expressing the CHMO- and CPMO-type BVMOs.<sup>[93]</sup> The BVMO collection allowed access to enantiocomplementary butyrolactones (Scheme 18) with optical pu-



**Scheme 18.** Chemoenzymatic preparation of chiral butyrolactones as starting material for the synthesis of biologically active compounds.<sup>[93]</sup> a) Zn, AcOH; b) microbial BVMO.

rities ranging from moderate to excellent. Biooxidation of *n*-butylcyclobutanone yielded exclusively the (*S*)-butyrolactone with good yields for all the BVMOs tested. In most cases conversion of aryl cyclobutanones led to (–)-lactones in good optical purities, with the enantiocomplementary lactones accessible via CHMO<sub>Brevi2</sub>. *p*-Chlorophenylbutyrolactone, an important intermediate in the preparation of GABA, was also obtained with high optical purities in both antipodal stereoisomers depending on the BVMO used. Another interesting substrate is piperonylcyclobutanone. The (–)-lactone was obtained in high enantioselectivity with most of the BVMOs tested, with the exception of the biocatalysts belonging to the CPMO-type clade (CPMO<sub>Coma</sub> and CHMO<sub>Brevi2</sub>). The antipodal (+)-lactone can be isolated with moderate selectivity by employing CHMO<sub>Brevi1</sub>. *E. coli* cells expressing HAPMO have also been employed in the biocatalyzed oxidation of prochiral 3-substituted cyclobutanones. For all compounds tested, moderate to good yields of the chiral butyrolactones were obtained. The stereoselectivity appears to be strongly dependent on the distance of the substituents to the carbonyl group. A phenyl group induces good enantioselectivity, whereas the presence of a benzyl group or branched alkyl chains led to a decrease in HAPMO selectivity.

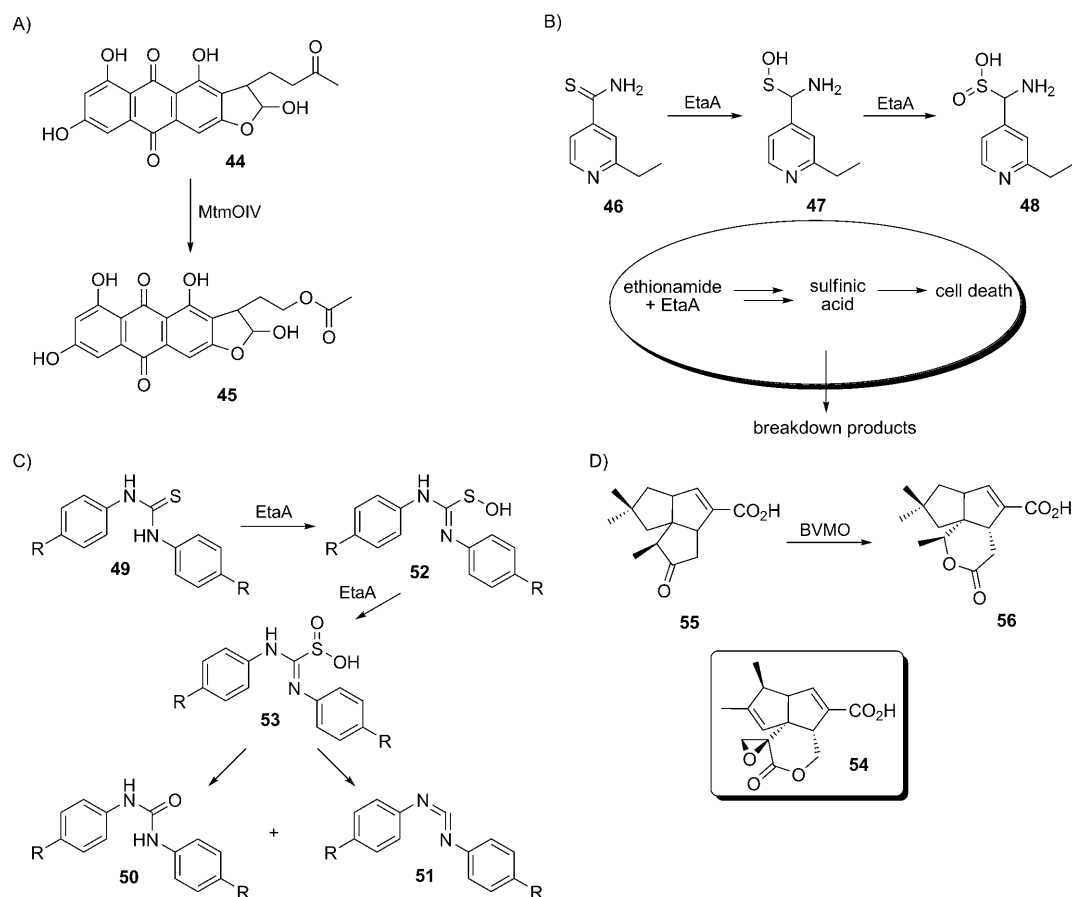
## 7. Baeyer–Villiger Monooxygenases in Newly Identified Metabolic Pathways

Mithramycin, an aureolic acid-type anticancer drug and calcium-lowering agent produced by *Streptomyces argillaceus* and other streptomycetes, contains a polyketide-derived tricyclin core. Its biosynthesis proceeds through the condensation of

multiple acyl-CoA units catalyzed by a type II polyketide synthase to form the intermediate premithramycinone. This precursor is glycosylated and C-methylated to afford premithramycin B (**44**), which undergoes an oxidative cleavage in its fourth ring, followed by decarboxylation and reduction of the 4'-keto group.<sup>[94]</sup> Experiments with isotopically labeled precursor have suggested that the key oxidative cleavage proceeds through a Baeyer–Villiger oxidation. To confirm this hypothesis, the monooxygenase MtmOIV was overexpressed, isolated, and employed as a biocatalyst in the conversion of premithramycin B.<sup>[95]</sup> The first step in the premithramycin B transformation was found to be the formation of the premithramycin B lactone (**45**; Scheme 19A), which confirms the role of MtmOIV as a BVMO. Overexpression of this enzyme was achieved by cloning the respective gene into an *E. coli* expression vector. Characterization of MtmOIV confirmed that it is a FAD and NADPH-dependent monooxygenase. The crystal structure of this BVMO has recently been determined by X-ray crystallography.<sup>[96]</sup> The MtmOIV structure shows significant differences when compared with the elucidated structures of CHMO from *Rhodococcus* sp. strain HI-31 and PAMO. This is in line with the fact that MtmOIV belongs to another flavoprotein monooxygenase class. In fact, it has been postulated that MtmOIV is a representative of a newly recognized class of BVMOs.

The  $\gamma$ - and  $\delta$ -lactones contribute to the flavor of many fruits and dairy products, which are produced from fatty acids by plants and microorganisms. These compounds were assumed to be stable end products, but further investigations have revealed that in the presence of the lactone-producing yeast *Sporobolomyces odor*, these final compounds were also transformed.<sup>[97]</sup> The catabolic pathway of such lactone degradation has been elucidated by employing labeled compounds. *S. odor* was shown to be able to degrade 4- and 5-hydroxydecanoic acids and esters by oxidation to the corresponding oxoacids or oxoesters, which are subsequently transformed by a Baeyer–Villiger oxidation. Thus, BVMO activity for a yeast has been described for the first time. Nevertheless, the responsible enzyme of this biotransformation has not yet been determined.

For decades, thioamides have been employed to treat tuberculosis infections. In 2000, it was first demonstrated that in *M. tuberculosis* the antimycobacterial activity of the most widely used thioamide, ethionamide (**46**), requires enzymatic activation.<sup>[98]</sup> Thus, it was recognized that S-oxygenation of the thiourea moiety of ethionamide was involved in its bioactivation to a toxic metabolite, which determines the therapeutic effect. The gene product Rv3854c (ethionamide monooxygenase, EtaA) of *M. tuberculosis* was identified as the enzyme re-



**Scheme 19.** Some examples of metabolic pathways in which BVMOs are involved: A) Baeyer–Villiger oxidation of premithramycin B into premithramycin B lactone catalyzed by MtmOIV.<sup>[95]</sup> B) EtaA-catalyzed activation of the prodrug ethionamide (**46**).<sup>[98]</sup> C) Activation of isoxyl (**49**) by a double oxidation catalyzed by EtaA and formation of the proposed activated form formimidamide **51**,<sup>[100]</sup> and D) PtIE-catalyzed Baeyer–Villiger oxidation of 1-deoxy-11-oxopentalenolactone in the metabolic pathway of pentalenolactone.<sup>[103]</sup>



sponsible for the bioactivation of ethionamide. Ethionamide monooxygenase was characterized as a FAD-containing enzyme capable of transforming ethionamide into two major products, the *S*-oxide **47** and 2-ethyl-4-amidopyridine. Ethionamide and its *S*-oxide present equivalent therapeutic potency and hepatotoxicity, whereas 2-ethyl-4-amidopyridine is relatively nontoxic and without therapeutic efficacy. Further oxygenation of the *S*-oxide to the sulfinic acid **48**, which spontaneously breaks down to 2-ethyl-4-amidopyridine and other products, is the postulated pathway for bioactivation of ethionamide in *M. tuberculosis* (Scheme 19B). The sulfinic acid, an unstable intermediate, is supposed to be the key target in the toxicity and has not been isolated. The EtaA sequence contains a BVMO-identifying fingerprint, which triggered a study to establish whether EtaA represent a bona fide BVMO. This revealed that it indeed is able to catalyze Baeyer–Villiger reactions with a wide set of aliphatic and aromatic ketones, and it also catalyzes enantioselective oxidation of methyl *p*-tolyl sulfide.<sup>[80]</sup> EtaA is also able to convert other prodrugs, such as isoxyl and thiacetazone, into reactive species against *M. tuberculosis*.<sup>[99]</sup> Jackson and co-workers have recently demonstrated that the EtaA-catalyzed oxidation of isoxyl **49** is required for this pro-drug to inhibit its lethal enzymatic targets in *M. tuberculosis*.<sup>[100]</sup> An analysis of the metabolites obtained from the in vitro transformation of isoxyl by isolated EtaA revealed the presence of a formimidamide **51** and the urea **50** derivative as final products, as shown in Scheme 19C. It has been proposed that the formimidamide is the activated form of isoxyl. The activation pathway is proposed to be triggered by EtaA-catalyzed successive sulfide oxidations, leading to compounds **52** and **53**. Interestingly, the Baulard group has shown that by targeting the regulator for EtaA expression, the transcriptional repressor EthR, with specific inhibitors, the efficacy of thioamide-based treatment can be improved.<sup>[98a]</sup>

Aflatoxins are a group of polyketide-derived secondary metabolites produced mainly by certain molds, for example, *Aspergillus flavus* and *Aspergillus parasiticus*. These compounds are highly toxic and carcinogenic in animals and humans. The biosynthetic pathway of aflatoxins has been extensively studied.<sup>[101]</sup> Various genes and corresponding enzymes involved in aflatoxin biosynthesis from coenzyme A have been described. It has been established that 25 genes involved in this biosynthetic feat, are clustered within a 70 kb DNA region in the chromosomes of *A. parasiticus* and *A. flavus*. Expression of most of these genes is regulated by the regulatory genes *afIR* and *afII*. Recently, the function of one of the genes (*moxY*) present in the aflatoxin gene cluster has been elucidated by disrupting the gene in *A. parasiticus* NRRL 2999. It was discovered that the *moxY* gene encodes the hydroxylversicolorone monooxygenase, which catalyzes the Baeyer–Villiger oxidation from hydroxyversicolorone to versiconal hemiacetal acetate, metabolites in the aflatoxins biosynthesis. This confirmed the role of a BVMO in this metabolic pathway.

The sesquiterpenoid antibiotic pentalenolactone (**54**), isolated from more than 30 *Streptomyces* species, possesses activity against both Gram-positive and Gram-negative bacteria as well as against pathogenic and saprophytic fungi. *Streptomyces aver-*

*mitilis* is a Gram-positive soil bacterium that produces a variety of secondary metabolites. Although pentalenolactone has not been detected in the organic extract of *S. avermitilis*, the shunt metabolite pentalenic acid has been isolated. This microorganism contains a 13.4 kb gene cluster containing 13 unidirectionally transcribed open-reading frames corresponding to the apparent biosynthetic operon for pentalenolactone.<sup>[102]</sup> One of these genes encodes a protein that contains the typical BVMO-identifying motif. In a recent study this protein, PtIE, was recombinantly produced and tested for conversion of 1-deoxy-11-oxopentalenic acid. The enzyme was supposed to catalyze the Baeyer–Villiger oxidation of this complex ketone into the corresponding lactone, pentalenolactone D.<sup>[103]</sup> However, although it was found that recombinant PtIE catalyzes a Baeyer–Villiger oxidation of 1-deoxy-11-oxopentalenic acid (**55**), the expected lactone was not formed. Instead, lactone **56** is formed in which the oxygen insertion is opposite to the expected outcome (Scheme 19D). This final product, neopentalenolactone D, represents a new branch of the pentalenolactone family tree.

*Gordonia* sp. strain TY-5 is an actinomycete capable of aerobic growth on gaseous propane as a carbon and energy source. This microorganism was able to use also acetone as source of carbon and energy. Recently, a study conducted to characterize acetone metabolism in *Gordonia* sp. at enzymatic and gene levels revealed the presence of two acetone-induced proteins. Their corresponding genes *acmA* and *acmB* encoded a Baeyer–Villiger monooxygenase and an esterase, respectively.<sup>[105]</sup> These enzymes enable the microorganism to transform acetone into methyl acetate, which is subsequently hydrolyzed to acetate and methanol. The *acmAB* gene cluster plays an important role in the metabolism of acetone derived from propane oxidation and clarifies the propane metabolic pathway of this *Gordonia* strain (propane → propan-2-ol → acetone → methyl acetate → acetic acid/methanol). Acetone monooxygenase (*AcmA*) was then purified revealing that it is a NADPH-dependent BVMO of 63 kDa showing activity towards cycloketones and aliphatic linear ketones.

The microbial genome sequences provide nowadays a wealth of information about new BVMOs. However, for effective genome mining for novel biocatalysts it is necessary to have tools for a reliable identification of genes that encode BVMOs. The above-mentioned BVMO-identifying sequence motif has proven to be very helpful in identifying novel BVMO genes.<sup>[10]</sup> The first example of a BVMO discovered by this approach was PAMO from *Thermobifida fusca*, which has been cloned, overexpressed in *E. coli* and employed for various selective Baeyer–Villiger reactions and sulfoxidations (vide infra).<sup>[53]</sup> The enzyme shows highest activity with aromatic compounds. Genes surrounding the *pamo* gene on the genome are predicted to play a role in degradation of aromatic compounds. This suggests that PAMO is involved in catabolism of aromatic compounds, possibly derived from lignin as *T. fusca* was recently suggested to participate in lignin degradation. The metabolic roles of BVMOs harvested in other genome-mining studies remain unclear. Nevertheless, Grogan and co-workers have convincingly shown by two BVMO discov-

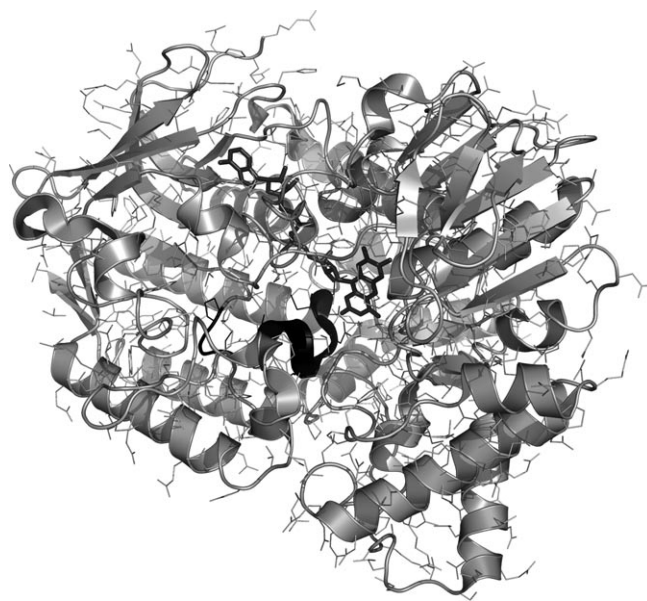
ery studies that the approach of mining genomes can be very powerful in obtaining valuable novel BVMOs.<sup>[105]</sup> By using ligation-independent cloning procedures,<sup>[106]</sup> 29 BVMO genes have been cloned from the genomes of *M. tuberculosis* and *Rhodococcus jostii* RHA1. Heterologous expression of soluble BVMOs in *E. coli* was achieved for about half of these genes. Whole cells expressing these genes have been screened for the oxidation of bicyclo[3.2.0]hept-1-en-6-one. This uncovered a wide diversity of regio- and/or enantioselective behavior. Analysis of the protein sequences of the *Rhodococcus* enzymes combined with the biocatalytic results has allowed grouping of these BVMOs as well as identifying group-specific protein sequences to BVMO. Such comparative analyses of sequence-related BVMOs are valuable to develop more reliable tools to predict catalytic properties of novel BVMOs.

## 8. Recent Methodologies for the Optimization of the BVMO-Biocatalyzed Processes

### Enzyme engineering

Nowadays, powerful methodologies exist to redesign enzymes to meet specific demands. Directed evolution approaches involve the (semi-)random mutagenesis of enzymes and subsequent screening for enzyme mutants with improved features. This approach has shown to be very effective to alter almost any enzyme characteristic. Only a limited number of BVMO redesign studies have been reported so far. The Reetz group was the first to report on the directed evolution of CHMO<sub>Acineto</sub>.<sup>[107]</sup> The gene encoding CHMO<sub>Acineto</sub> was subjected to error-prone PCR, after which relatively small mutant libraries (10 000) were screened for mutant CHMOs that displayed improved enantioselectivity for 1) Baeyer–Villiger oxidation of 4-hydroxycyclohexanone, and 2) sulfoxidation of methyl *p*-tolyl sulfide. Oxidation of prochiral 4-hydroxycyclohexanone results in formation of the corresponding seven-membered lactone, which spontaneously rearranges to form the final hydroxy butyrolactone with no change in stereochemistry. The wild-type CHMO<sub>Acineto</sub> only shows 9% *ee* for formation of the *R* enantiomer. Upon screening 10 000 mutants, 24 mutants were shown to have an increased *R* selectivity whereas 12 displayed reversal of enantioselectivity. Optimization of the *R* selectivity by a second round of epPCR allowed creation of a quadruple mutant that catalyzed the Baeyer–Villiger oxidation with 90% *ee*. When wild-type CHMO<sub>Acineto</sub> is used for oxidation of prochiral methyl *p*-tolyl sulfide the (*R*)-sulfoxide was obtained with only 14% *ee*. Several mutants were found from the same library as above with significantly improved enantioselectivity (*ee* values > 95%), some being *R* and others being *S* selective.<sup>[108]</sup> Interestingly, one of the best mutants for enantioselective sulfoxidation was F432S whereas mutations of F432 were also found when looking for mutants with improved enantioselectivity for oxidation of 4-hydroxycyclohexanone. In a subsequent study, a set of the generated CHMO<sub>Acineto</sub> mutants was selected, which displayed enantiocomplementary for the Baeyer–Villiger reaction of other structurally different ketones such as prochiral 3-substituted cyclobutanones, 4-substituted cyclohexanones,

and bi- and tricyclic ketones.<sup>[109]</sup> For almost all the substrates, mutants that contained a F432 mutation had a strong effect on the biocatalytic properties of CHMO. Therefore this residue can be regarded as a “hot spot” for tuning selectivity of CHMO<sub>Acineto</sub>. Identification of such hot spots offers the possibility to focus enzyme-engineering efforts. Focusing the randomization in mutant libraries can also be inspired by analysis of enzyme structures. An example of this is the study in which CPMO<sub>Coma</sub> has been also subjected to mutagenesis in order to find new mutants with improved enantioselectivities.<sup>[110]</sup> By comparison of a homology model of this enzyme with the crystal structure of PAMO several residues were selected for preparing mutant libraries. By this approach, mutants were obtained that showed significant improvement in enantioselectivity with 4-substituted cyclohexanones.<sup>[77]</sup> Another even more focussed engineering effort has been the structure-inspired site-directed mutagenesis of PAMO. By targeting several active-site residues by site-directed mutagenesis, the M446G mutant was discovered that exhibits a significantly changed substrate spectrum and improved enantioselectivity for, for example, sulfoxidation reactions. It was also the first example of a BVMO that is able to convert indole into indigo blue.<sup>[56]</sup> Reetz has focused mutagenesis efforts in the same structural area, the loop region 440–447 in PAMO (which also includes the position of F432 in CHMO), to alter the catalytic properties of PAMO (Figure 2).<sup>[111]</sup> First, deletion mutants were made to enlarge the active site; this resulted in mutants that displayed activity towards more bulky ketones. Subsequent semi-random mutagenesis studies have targeted several residues in this loop region revealing that this part of the protein is crucial in forming the substrate-binding site. In a recent study, mutations that are relatively far from the active site of PAMO and on the surface of the protein have been engineered that still affected the catalytic behavior.<sup>[112]</sup> The fact that distant mutations in



**Figure 2.** Crystal structure of PAMO with the FAD cofactor in sticks and the 440–447 loop region in dark gray.

PAMO can affect enzymatic properties might reflect the dynamic events that are involved in BVMO-mediated catalysis. The recently elucidated structures of CHMO from *Rhodococcus* sp. strain HI-31 and the PAMO structural and kinetic data indicate that during the catalytic cycle of these BVMOs, major conformation changes occur.<sup>[115,16]</sup> For more focused BVMO redesign studies, it will be crucial to obtain a better understanding of this dynamic behavior.

Whereas the engineering efforts above focussed on altering the substrate specificity and enantioselectivity, some BVMO engineering has also been performed to alter the coenzyme specificity. Unfortunately, these site-directed mutagenesis studies have not yet resulted in a BVMO that is efficient with NADH.<sup>[113]</sup>

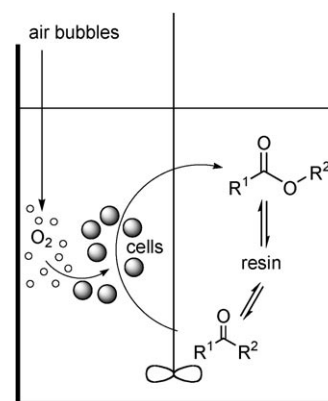
### Scaling up methodologies

The use of Baeyer–Villiger monooxygenases for synthetic purposes is highly attractive when compared with the conventional chemical Baeyer–Villiger oxidations.<sup>[114]</sup> Apart from the high chemo-, regio-, and/or enantioselectivities of BVMOs, the operational conditions for BVMO-mediated conversions are mild and environmentally friendly with air as oxidant. However, the implementation of BVMOs at an industrial level is not yet well developed. One of the major hurdles is the fact that BVMOs depend on the reduced nicotinamide coenzyme NADPH for activity. One of the easiest ways of dealing with this requirement is the use of whole cells. In such fermentation set-up one relies on the NAD(P)H recycling activity of metabolically active cells. This is typically achieved by feeding the cells with a cheap carbon source together with the target substrate.

However, employing the wild-type strain often presents disadvantages. In the case of CHMO<sub>Acinetor</sub> these are low activities, handling of a pathogen and the presence of undesired side reactions. For these reasons, the implementation of whole-cell processes has been facilitated by the use of recombinant cells overexpressing the desired enzymes.<sup>[64b,115]</sup> Host strains ideally exhibit fast growth rates, lack interfering activities, and support efficient coenzyme regeneration. *E. coli* strains fulfil these requirements and have been used for whole-cell transformations. However, a number of limitations have been identified when analyzing the biooxidation of the model substrate bicyclo-[3.2.0]hept-2-en-6-one by recombinant *E. coli*.<sup>[116]</sup> The reaction is inhibited by the substrate at concentrations higher than 1.0 g L<sup>-1</sup> and by the final products at levels of 3.5 g L<sup>-1</sup>. Furthermore, the biocatalyst concentration and the oxygen supply were found to be bottlenecks. The effect of the biocatalyst concentration has been studied by employing fresh *E. coli* TOP10 cells expressing CHMO<sub>Acinetor</sub>. At low cell concentrations, there was a linear increase in the initial reaction rate, but once a certain maximum rate was achieved, a steady decrease in the rate was observed as a function of the cell concentration. Because molecular oxygen is required not only as metabolic substrate for cell maintenance but also for the biocatalyzed oxidation, when the cell concentration is high and oxygen becomes a limiting factor, oxygen is used preferentially by *E. coli* for its metabolism.<sup>[117]</sup> Thus, oxygen-limited conditions led to a dra-

matic decrease in space–time yields. Methods for enhancing oxygen mass transfer consist in a longer bubble residence time, the formation of smaller air bubbles to increase the interface area or the use of oxygen-enriched aeration. Except for finding an effective oxygen supply, also tuning the metabolic state of the cells is of importance. For this reason, nongrowing conditions were initially developed for *E. coli* cells expressing CHMO<sub>Acinetor</sub> by growing the cells until the stationary phase.<sup>[118]</sup> After that, cells are harvested and resuspended in a minimal salt medium lacking a nitrogen source. In these conditions, the cell replication is inhibited while the cellular machinery is still able to regenerate NADPH and to act as a catalyst.<sup>[119]</sup> Nongrowing conditions have recently been employed in biooxidations catalyzed by *E. coli* whole cells expressing CHMO<sub>Acinetor</sub> CPMO<sub>Comar</sub> and some of their mutants. The scaling up of the reactions under these conditions led to a high increase in the conversions for several 4-substituted cyclohexanones. In addition, the use of resting cells gave the optically active lactones in good yields for a number of substrates for which no reaction was previously observed. By this it was possible to obtain both enantiomeric lactones depending on the biocatalyst employed. The conversion efficiency was superior when compared with the use of growing cells. This can be explained by a better balance of BVMO activity and other metabolic activities and a more effective substrate transport in nongrowing cells due to an increased membrane permeability caused by a high concentration of hydrophobic substrate and product in the reaction medium.

A significant improvement in conversion efficiency was finally developed by the resin-based in situ substrate-feeding product-removal method (SFPR, Figure 3).<sup>[120]</sup> This methodology employed an adsorbing polymeric resin that acts as a reservoir for the starting material and as a trap for the final product. The substrate is loaded into the reactor before performing the bio-transformation. It diffuses into the reaction medium and is transformed by the added recombinant cells, whereas the formed product will be readsorbed onto the same resin. The system is conveniently tuned by a proper selection of the substrate/resin ratio. Thus, both the substrate and product concentrations are controlled in order to maintain them below their inhibitory or toxic levels and allow a higher productivity.



**Figure 3.** The in situ substrate-feeding product-removal (SFPR) concept applied to the Baeyer–Villiger oxidation of ketones catalyzed by BVMOs.<sup>[121]</sup>

SFPR methods have been described previously, but Hilker et al. have applied the concept to a BVMO-catalyzed oxidation for the first time: the Baeyer–Villiger reaction of the racemic ketone ( $\pm$ )-bicyclo[3.2.0]hept-2-en-6-one by CHMO<sub>Acinetor</sub>.<sup>[121]</sup> For optimization, 14 resins were tested by looking for an adsorbent with a high capacity at the equilibrium concentration for the biotransformation. The best results were obtained with the Dowex Optipore L493 resin. Product can be recovered from the resin by washing with a polar solvent or by Soxhlet extraction. The resin can be reused after washing with methanol and water. Three types of reactors, one conventional, a recycle reactor, and a bubble column reactor were studied and compared. The best one proved to be the bubble column, with which 25.0 g (0.23 mol) of starting ketone was completely oxidized by using a one-liter vessel with a volumetric productivity of about  $1.0 \text{ g L}^{-1} \text{ h}^{-1}$ . This led to nearly exclusive production of the two corresponding regioisomeric lactones with high yield and excellent enantiomeric excess ( $ee > 98\%$ ). An additional benefit of an in situ SFPR methodology is the opportunity to continue the biotransformation even in case of loss of cell activity. As most of the substrate and product are stored in the resin, exhausted cells can be replaced by fresh ones by simply decanting the resin. Thus, the use of SFPR systems enabled the non-natural substrate toxicity to expression hosts, enzyme inhibition, and biocatalyst stability to be overcome as the most critical aspects of BVMO-mediated biotransformations.

The SFPR approach has been also employed for the oxidation of three ketones employing *E. coli* cells expressing CPMO<sub>Coma</sub>.<sup>[122]</sup> The model reaction was the oxidation of prochiral 4-methylcyclohexanone to the corresponding lactone. First, the optimal substrate concentration for this reaction was determined ( $3.4 \text{ g L}^{-1}$ ). The optimization of other parameters resulted in the use of a reduced oxygen saturation 50–30%, and the addition of glucose ( $4.0 \text{ g L}^{-1}$ ) after induction of protein production. Also the addition of a  $\beta$ -cyclodextrin was beneficial because it improves the substrate transport into the cell and/or serves as a reservoir for the substrate within the fermentation broth. In the best reaction conditions, a total yield of 84% was achieved. Again, the use of nongrowing conditions also resulted in higher yields with respect to the biotransformation in growing conditions. Subsequently, preloaded resin Lewatit VPOC 1163 was used to develop the SFPR processes. By using this,  $10 \text{ g L}^{-1}$  of 4-methylcyclohexanone can be converted by CPMO-growing cells with a 77% yield after 26 h, whereas the use of nongrowing conditions allowed to transform  $15 \text{ g L}^{-1}$  of the same substrate with 86% yield after 20 h of fermentation time. The latter represents a fivefold increase in substrate concentration and an improved yield compared with the results from growing-cell systems without the SFPR methodology. This study was then extended to the biooxidation of 3-methylcyclohexanone which was regioselectively oxidized to the proximal lactone. Reaction with  $15.0 \text{ g L}^{-1}$  of this ketone led to 80% yield after 10 h. 8-Oxabicyclo[3.2.1]oct-6-en-3-one can be oxidized to the corresponding lactone, an interesting and versatile intermediate. Previous attempts to perform the whole-cell biotransformation of this ketone were developed at

$2.0 \text{ g L}^{-1}$ , as higher concentrations elicited product inhibition. SFPR represented an effective alternative to the preparation of this interesting lactone: after 22 h a 78% isolated yield was achieved. SFPR has recently also been employed for enantioselective kinetic resolution of the aromatic ketone, ( $\pm$ )-3-phenyl-2-butanone.<sup>[123]</sup>

The examples above show that the SFPR approach can be very effective. Nevertheless, some SFPR-related limitations have to be considered, for example, limitations due to the substrate/product couple. The adsorption/desorption equilibrium on a given resin depends on the physicochemical properties of the compounds, so they have to be compatible with the demanded working concentrations. Also, not every substrate or product will easily cross the cell membrane, and this prohibits the use of whole cells.

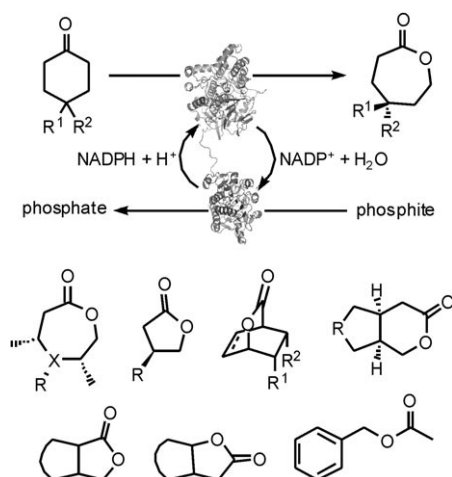
The alternative approach by immobilizing recombinant whole-cell expression systems was proposed very recently and certainly requires future attention based on favorable initial results.<sup>[124]</sup>

### Self-sufficient biocatalysts

Whole (recombinant) cells are frequently used in industry for biocatalytic processes. However, in particular cases other approaches are necessary, for example, when substrate and/or product are toxic to cells or cannot pass the cell membrane. To circumvent such problems the use of isolated enzymes might be a solution. This approach presents some advantages, as affording clean, effective, and simple reactions. But, when dealing with redox enzymes, these processes typically require redox coenzymes. In case of BVMOs, the relatively unstable nicotinamide coenzyme NAD(P)H is essential for BVMO activity. Because NADPH is an expensive compound, it cannot be applied stoichiometrically. Therefore, several coenzyme regeneration methods that allow the catalytic use of NADPH have been developed. A good NADPH regeneration system should meet several criteria such as low cost, no risk of byproducts, and no interference with the biocatalytic properties of BVMOs. The most frequently employed approach includes utilization of isolated enzymes by coupling a second enzymatic system (glucose-, glucose-6-phosphate, formate, phosphite or alcohol dehydrogenases) to the BVMO-catalyzed oxidation.<sup>[125]</sup> The dehydrogenase will regenerate NADPH at the expense of a sacrificial substrate, for example, glucose. Recently, this approach was refined in our labs by fusing a dehydrogenase to individual BVMOs (Scheme 20).<sup>[126]</sup> Fusion proteins have been exploited in the last years for protein purification, but have not been used extensively for biocatalytic purposes.<sup>[127]</sup> The recently described phosphite dehydrogenase (PTDH) was selected as a fusion partner for BVMOs.<sup>[128]</sup> This bacterial enzyme catalyzes oxidation of phosphite into phosphate in a quasi-irreversible process during which it reduces  $\text{NADP}^+$  to NADPH. Initially, three fusion biocatalysts (CRE/BVMOs) were prepared by using PAMO, CHMO<sub>Acinetor</sub> and CPMO<sub>Coma</sub> as fusion partners for PTDH.

Analysis of the created CRE/BVMOs revealed that fusing the BVMOs with PTDH did not significantly alter their catalytic properties or thermostability. Also, no inhibition of either the





**Scheme 20.** The concept of using a fused BVMO (orange/top) and PTDH (magenta/bottom) as biocatalyst. Such CRE/BVMO system supports effective coenzyme regeneration. Some of the compounds that were prepared with CRE/BVMOs are shown in the lower panel.<sup>[126, 130]</sup>

BVMOs or PTDH by the involved substrates or products was observed. CRE/CHMO and CRE/CPMO were applied as whole cells or as crude cell-free extracts in enantioselective oxidations by using prochiral as well as racemic ketones as starting materials. This revealed that the stereoselectivity of the fused BVMOs was not affected. The data also showed that the fusion of PTDH as a coenzyme-regenerating enzyme does not interfere with BVMO functioning. As an appealing side note, preparative biotransformations can be carried out by using crude cell-free extract without addition of NADP<sup>+</sup> because sufficient quantities of the coenzyme seem to be present in *E. coli* cells; this represents a significant simplification in the experimental protocol and potential future development of these biocatalysts.

One drawback of the CRE/BVMOs was the relatively poor stability of the PTDH subunit. To solve this, a second generation of self-sufficient biocatalyst was prepared involving three important modifications: 1) a thermostable mutant of PTDH for effective coenzyme regeneration was used as N-terminal fusion partner,<sup>[129]</sup> 2) a codon optimized PTDH gene for improving the expression in *E. coli* was used, and 3) a sequence for a histidine purification tag at the free N terminus of PTDH was integrated in the expression vector. Six 2nd generation fusion proteins (CRE2/BVMOs) were prepared by using CPMO, CHMO, HAPMO, EtaA, PAMO, and its M446G mutant.<sup>[130]</sup> Again, the purified CRE2-BVMOs display similar kinetic parameters when compared with the nonfused enzymes. The fused biocatalysts have been employed as cell-free extracts for the conversion of bicyclo[3.2.0]hept-2-en-6-one, which revealed that the robustness of these CRE2/BVMOs does not depend anymore on PTDH stability.

### Other advances in regeneration systems

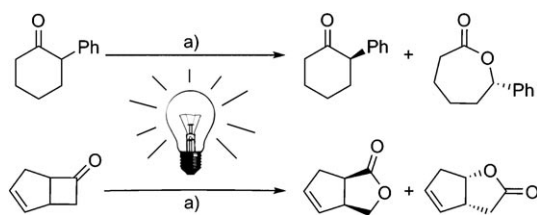
In the last few years, some methodologies have been developed to circumvent the need for nicotinamide coenzymes in BVMO-catalyzed reactions. These methods aim at direct regen-

eration of the flavin cofactor of BVMOs and involve chemical or the photochemical cofactor regeneration.

The rhodium complex [Cp\*Rh(bpy)(H<sub>2</sub>O)]<sup>2+</sup>, first synthesized by Steckhan and co-workers,<sup>[131]</sup> has been widely applied for the reduction of flavins, porphyrins, and nicotinamides and has been employed as a regeneration reagent in reactions catalyzed by oxidoreductases.<sup>[132]</sup> In the presence of a formate salt, this complex leads to [Cp\*Rh(bpy)H]<sup>+</sup>, which is able to act as an electron donor capable of reducing a flavin cofactor. Thus, [Cp\*Rh(bpy)(H<sub>2</sub>O)]<sup>2+</sup> has been employed in the enzymatic oxidation of prochiral sulfides by using PAMO, HAPMO, CHMO<sub>Acinetor</sub> and EtaA.<sup>[133]</sup> When the complex was employed as coenzyme substitute, it was able to directly reduce the enzyme-bound FAD, but the activity and selectivity displayed by the BVMOs under these conditions were very low. The presence of NADP<sup>+</sup> in the reaction medium could restore the activity and selectivity of the BVMOs to some extent. Nonetheless, using chemical regeneration with these reactions led to worse results than when employing the enzymatic regeneration of NADPH due to inefficient delivery of reducing equivalents. The results confirmed the dual role of the NADP<sup>+</sup> coenzyme: it serves as an electron donor while it also is part of the active site during the oxidation reaction. Kinetic and structural data have shown that the NADP<sup>+</sup> stays bound throughout the catalytic cycle after flavin reduction until it is replaced by NADPH. The bound NADP<sup>+</sup> exerts a positive effect on the stability of the peroxyflavin intermediate and also is crucial for enantioselectivity, presumably by direct or indirect interaction with the bound substrate. Therefore, for a fully effective and selective BVMO, a catalytic amount of NADP<sup>+</sup> will always be advantageous.

Recently, an attractive photochemical approach has been described by Hollman et al. for the flavin regeneration in BVMO-biocatalyzed reactions, with the aim of avoiding the use of the expensive nicotinamide coenzyme.<sup>[134]</sup> Photochemical regeneration of cofactors is a highly attractive approach because it enables the exploitation of solar light as a cheap and environmentally friendly reagent. To generate light-driven electron transfer, photosensitive material is required such as organometallic complexes, flavins, and semiconductors. Such compounds promote electron generation by using suitable electron donors, for example, EDTA or mercaptoethanol (Scheme 21). The PAMO-P3 mutant has been tested as a biocatalyst in the enzymatic oxidation of prochiral ketones by using solar light, EDTA, and an additional flavin as electron mediator. As could be expected (vide supra), in the absence of nicotinamide coenzyme, no enzymatic oxidation was observed. Addition of small amounts of NADP<sup>+</sup> afforded the conversion of 2-phenylcyclohexanone into (S)-2-phenylcaprolactone with excellent enantiomeric excess (*ee* = 97%) and 48% conversion, showing that the selectivity of this BVMO is not altered by the regeneration conditions. This system has also been tested in the oxidation of bicyclo[3.2.0]hept-2-en-6-one with high selectivity (*ees* higher than 90%).

It was also shown that the added FAD can be replaced by riboflavin or FMN, which demonstrates that no significant exchange of reduced FAD with the protein-bound FAD occurs.



**Scheme 21.** Enzymatic Baeyer–Villiger oxidation of ketones employing light as cofactor-regeneration system.<sup>[134]</sup> a) PAMO-P3/Tris-HCl, EDTA/NADPH/FAD.

This supports a catalytic role of the free flavin in the reduction of the enzyme-bound cofactor. No evidence was found for photoreduction of NADP<sup>+</sup>. The major disadvantage of this photochemical regeneration system is that the efficiency is significantly lower than that observed when employing conventional regeneration methods. In more recent studies, it was shown that the photochemical regeneration is limited by the O<sub>2</sub>-dependent uncoupling of the regeneration systems from the oxidation reaction and by the slow transfer between the free and the BVMO-bound flavin.<sup>[135]</sup> To overcome the first drawback, covalent attachment of the free flavin to the enzyme surface via a linker can be considered, whereas for the second limitation, smaller electron shuttle molecules or PAMO variants with higher accessibility to the enzyme-bounded flavin might be employed. Also the use of deazaflavins as redox mediators might prevent the uncoupling reactions. In fact, a recent study employing deazaflavins as mediators for the photochemical regeneration of the P450 monooxygenase BM3 from *Bacillus megaterium* in the hydroxylation of C–H bonds lead to an efficient process under aerobic conditions.<sup>[136]</sup>

## Summary

During the past years, a highly complementary platform of BVMOs has become available for diverse oxygenation reactions such as desymmetrizations, regiodivergent transformations, and (dynamic) kinetic resolutions. Individual representatives of the available BVMO collection are able to operate on functionally and structurally diverse substrates (cycloketones, aromatic ketones, linear ketones, and sulfides). It is possible to access both enantiomers of a lactone, ester or sulfoxide in optically pure form and good synthetic yields on a large number of compounds. Preparative-scale biotransformations have been realized by using purified enzymes or recombinant whole-cell systems with both approaches offering certain pros and cons depending on the given conversion. In many cases, the enzyme-mediated Baeyer–Villiger oxygenation provides access to optically pure lactones that are impossible to obtain by conventional chemical methods. Although versatile, BVMOs are also highly chemoselective. Except for representing valuable biocatalytic tools, BVMOs have also been shown to be crucial in several biological processes. Catabolic routes and the synthesis of specific secondary metabolites have been shown to fully depend on a particular BVMO activity. Of special interest is the exploitation of BVMOs as prodrug targets. *M. tuberculosis* infections have been often treated with a range of thiourea

and thioamide-containing prodrugs for decades. The antitubercular effect of these prodrugs is due to activation by a BVMO.

For performing effective BVMO-mediated catalysis several technical issues need to be addressed. One prerequisite for optimal BVMO activity is to supply the enzyme with sufficient reducing equivalents, typically by using NADPH. Several novel approaches for NADPH regeneration have been developed in recent years. By using proper conditions, whole recombinant cells provide an efficient format to perform catalysis as the intracellular machinery is able to recycle NADP<sup>+</sup>. The use of whole cells has been integrated with the utilization of resins or a second phase that enables the use of higher substrate concentrations and relatively easy work-up procedures. An alternative approach for coenzyme recycling was achieved by fusing BVMOs to a coenzyme-regenerating enzyme. These self-sufficient fusion proteins were shown to be as effective in recycling NADPH, and they retain full BVMO activity and selectivity. Another attractive and novel approach for providing reducing equivalents has been the finding that light can be used for this. By using several additives in combination with light, BVMOs could be used as biocatalysts without the need to recycle NADP<sup>+</sup>.

## Outlook

Enzyme-mediated Baeyer–Villiger oxygenations are still a developing field. With growing information on the structure of the involved biocatalysts and their dynamic behavior upon biotransformation, it gradually becomes possible to predict and modify the catalytic performance of BVMOs. Still, crucial aspects (enzyme stability, improved efficiency) have to be addressed and solved in a satisfactory manner, to make this enzyme class even more appealing for future application on industrial scale. A variety of tools provided by molecular biology have already been successfully applied to further improve the capabilities of this enzyme group to be tailored to the needs and expectations of modern synthesis. Except for discovery or design of a proper BVMO, development of BVMO-based applications will also depend on efficient methodologies to support optimal catalysis. A key issue is the coenzyme dependency of BVMOs. Although the use of whole recombinant cells might be a solution for a number of target reactions, it will not always be applicable. Therefore, developing or improving novel approaches that involve the use of isolated enzymes is still an interesting target for further research. Additional effort will be required and might very well be expected in the immediate future to ultimately establish enzyme mediated Baeyer–Villiger oxidation as an attractive process in asymmetric organic synthesis from lab-scale to industrial production.

## Acknowledgements

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**Keywords:** Baeyer–Villiger monooxygenases • biocatalysis • coenzyme regeneration • prodrugs • sulfoxidation

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